EXHIBIT

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Cecil Czerkinsky
Fabienne Anjuere
Jerry R. McGhee
Annie George-Chandy
Jan Holmgren
Marie-Paule Kieny
Kohtaro Fujiyashi
Jiri F. Mestecky
Valérie Pierrefite-Carle
Carola Rask
Jia-Bin Sun

Mucosal immunity and tolerance: relevance to vaccine development

Authors' addresses
Cecil Czerkinsky[1], Fabienne Anjuere[1], Jerry R. McGhee[2], Annie George-Chandy[1,3], Jan Holmgren[3], Marie-Paule Kieny[4], Kohtaro Fujiyashi[2], Jiri F. Mestecky[2], Valérie Pierrefite-Carle[1], Carola Rask[3], Jia-Bin Sun[3],
[1]INSERM Unit 364, Faculté de Médecine-Pasteur, Nice, France.
[2]Department of Microbiology and The Immunobiology Vaccine Center, The University of Alabama at Birmingham, Birmingham, Alabama, USA.
[3]Department of Medical Microbiology and Immunology, University of Göteborg, Göteborg, Sweden.
[4]INSERM Unit 74, Institute of Virology, Strasbourg, France.

Correspondence to:
Cecil Czerkinsky
INSERM Unit 364
Faculté de Médecine-Pasteur
Nice
France
Fax. 33 493 81 9445
e-mail: czerkinsky@dial-up.com

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Summary: The mucosal immune system of mammals consists of an integrated network of lymphoid cells which work in concert with innate host factors to promote host defense. Major mucosal effector immune mechanisms include secretory antibodies, largely of immunoglobulin A (IgA) isotype, cytotoxic T cells, as well as cytokines, chemokines and their receptors. Immunologic unresponsiveness (tolerance) is a key feature of the mucosal immune system, and deliberate vaccination or natural immunization by a mucosal route can effectively induce immune suppression. The diverse compartments located in the aerodigestive and genitourinary tracts and exocrine glands communicate via preferential homing of lymphocytes and antigen-presenting cells. Mucosal administration of antigens may result in the concomitant expression of secretory immunoglobulin A (S-IgA) antibody responses in various mucosal tissues and secretions, and under certain conditions, in the suppression of immune responses. Thus, developing formulations based on efficient delivery of selected antigens/tolerogens, cytokines and adjuvants may impact on the design of future vaccines and of specific immunotherapeutic approaches against diseases associated with untoward immune responses, such as autoimmune disorders, allergic reactions, and tissue-damaging inflammatory reactions triggered by persistent microorganisms.

Introduction

The mucous membranes covering the aerodigestive and the urogenital tracts as well as the eye conjunctiva, the inner ear and the ducts of all exocrine glands are endowed with powerful mechanical and physicochemical cleansing mechanisms that degrade and repel most foreign matter. In addition, a large and highly specialized immune system protects these surfaces and thereby also the body interior against potential insults from the environment. In a healthy human adult, this local immune system contributes almost 80% of all immunocytes. These cells are accumulated in or in transit between various mucosal organs and glands and together they form the mucosa-associated lymphoid tissue (MALT), the largest mammalian lymphoid organ system.

The MALT has three main functions: 1) to protect the mucous membranes against colonization and invasion by potentially dangerous microbes encountered; 2) to prevent

uptake of undegraded antigens including foreign proteins derived from ingested food and commensal microorganisms; and 3) to prevent the development of harmful immune responses to these antigens if they do reach the body interior. At variance with the systemic immune apparatus, which functions in a normally sterile milieu and often responds vigorously to invaders, the MALT guards organs that are replete with foreign matter. It follows that upon encounter with this plethora of antigenic stimuli, the MALT must ignore such antigens, or economically select appropriate effector mechanisms and regulate their intensity to avoid bystander tissue damage and immunological exhaustion. Since this system cannot discriminate between pathogenic microorganisms on the one hand, and innocuous matter, such as dietary matter and commensal microorganisms, on the other, it is likely to recognize all foreign peptides. Elucidating the mechanisms that determine whether recognition of such diverse antigens will result in active immunity, immune suppression or ignorance, will have a major impact in the development of effective mucosal vaccines against infectious and inflammatory diseases.

Although the immune apparatus is remarkably diverse, there is strong evidence that certain types of immune responses take place and are basically restricted to certain anatomic locations within the body. The MALT represents a well-known example of such a compartmentalized immunological system. As opposed to the central and peripheral lymphoid organs, the MALT contains inhomogeneously distributed B and T cells whose phenotype, repertoire, developmental origin, secretion products and hence probably also function are different. First, the prime immunoglobulin isotype produced and assembled in mucosal tissues is secretory immunoglobulin A (IgA), which is only present in trace amounts in the intravascular compartment. Further, and although major species differences have been observed, non-conventional lymphocytes rarely seen in the spleen and peripheral lymph nodes are encountered at appreciable frequency in different mucosal locations, e.g. α/β TCR$^+$CD4$^-$CD8$^-$ cells, γ/δ TCR$^+$CD4$^-$CD8 α^+CD8 β^- and γ/δ TCR$^+$CD4$^-$CD8 α^-CD8 β^- T cells in the gut epithelium. The antigen receptor repertoire is also different in each location. Thus, self-reactive T cells are detected among murine intestinal intraepithelial α/β and γ/δ TCR$^+$ T lymphocytes. Furthermore, in contrast to the T and B lymphocytes found in central and peripheral lymphoid organs, certain γ/δ and α/β T cells found in the mucosa, and presumably also mucosal CD5$^+$ B cells, do not depend on the thymus or bone marrow for their development, respectively. In addition to divergent lineages that are driven to different organs by tissue-specific homing receptors, local selection mechanisms may be important in the diversification of mucosal immune responses. Selection may be exerted by local antigens, antigen-presenting cells (APCs) whose function varies in each anatomical location, cytokines, chemokines and cell-matrix interactions, thus leading to the expansion and maintenance of some clones, whereas others are diluted out or deleted. Thus, by its cellular composition, and through the compartmentalization of its afferent and efferent limbs, the MALT functions essentially independently of the systemic immune apparatus.

Although most infectious microorganisms colonize or enter the host through mucosal membranes, it is the systemic immune system which has been the focus of most vaccine research. Aside from the ease of their administration, an important advantage of mucosal vaccines is the theoretical possibility of inducing both mucosal and systemic immune responses, while the reverse does not hold true. Since specific humoral immune defense is provided by both serum and by secretory antibodies, predominately of the IgA isotype, that are selectively transported into external secretions, most vaccines considered for further improvement or development should ideally induce both systemic and mucosal responses.

Equally important to host immune defense is the capacity of MALT to promote specific immunological unresponsiveness (tolerance) after natural or deliberate mucosal exposure to a variety of antigens. This form of tolerance is considered as a major adaptive immune defense mechanism whereby we avoid developing harmful immune responses against the plethora of dietary and airborne antigens encountered each day.

Thus, from a theoretical standpoint, the possibility of manipulating the mucosal immune system towards positive immunity and/or tolerance appears extremely attractive when considering strategies aimed at protecting the host from colonization or invasion by microbial pathogens but also to prevent and/or to modulate the development of potentially harmful immunological reactions against the same pathogens and also against certain self antigens and allergens. Such considerations should finally bring mucosal vaccines to center stage in vaccine development.

Adaptive effector mechanisms in mucosal immune defense

Three major adaptive effector mechanisms participate in the immune defense of mucosal surfaces. Secretory antibody formation and antigen-specific cell-mediated cytotoxicity are the primary mechanisms involved in antimicrobial defense and act primarily but not exclusively in the epithelium of all mucosal tissues. In addition, a third form of mucosal immune defense is contributed by regulatory cells which act in both epithelium

and submucosa as well as in extramucosal tissues mainly through the production of soluble mediators. Although these cells may regulate IgA antibody formation and the development of cell-mediated cytotoxic responses, they participate in the maintainance of mucosal tolerance against most environmental matters and as such can be regarded as key players in mucosal defense against inflammation.

Secretory IgA

Immune responses expressed in mucosal tissues are typified by secretory immunoglobulin A (S-IgA) antibodies. S-IgA constitutes the predominant Ig class in human external secretions, and is the best known entity providing specific immune protection for mucosal tissues. While the synthesis and assembly of J chain-containing polymeric IgA molecules is contributed to by plasma cells, mucosal epithelial cells synthesize a secretory component which acts as a cell surface receptor to facilitate the transport of newly formed IgA across epithelial cells. The resistance of S-IgA against endogenous proteases makes antibodies of that isotype uniquely well suited to protect mucosal surfaces. S-IgA antibodies provide "immune exclusion" of bacterial and viral pathogens, bacterial toxins and other potentially harmful molecules, a function that appears to be facilitated by its affinity for binding to mucus. S-IgA has also been reported to neutralize directly a number of viruses (1, 2), to mediate antibody-dependent cell-mediated cytotoxicity, and to interfere with the utilization of growth factors for bacterial pathogens in the mucosal environment.

Mucosally derived S-IgA differs from bone marrow-derived serum IgA, not only in terms of specific antibody activity but also in the proportions of polymeric vs monomeric forms (3). The ontogenies of the mucosal and systemic IgA compartments display characteristic and apparently independent patterns of maturation. Adult levels of S-IgA are reached in external secretions considerably earlier (1 month to 2 years) than in the serum (adolescence) (4, 5). Experiments that addressed the origin of mucosal antibodies have indicated that an overwhelming proportion of such antibodies are produced locally in mucosal tissues and that only a minor fraction derive from the circulation in most species, including humans (6, 7).

Mucosal cytotoxic T lymphocytes

Although the focus of most investigations has been on mucosal cytotoxic T lymphocytes (CTLs) induced by viruses, it should be borne in mind that antibody-mediated cytotoxicity and natural killer (NK)-cell activity are major responses associated with mucosal lymphocytes (8–14). The relevance of the latter responses in the field of mucosal vaccine development has not been ascertained and is therefore beyond the scope of this review.

Two major effector mechanisms associated with CTLs have been described: exocytosis from CTLs onto target cells of granules containing the pore-forming protein perforin and several serine proteases also termed granzymes, and ligation of a tumor necrosis factor (TNF) receptor-like molecule (Fas or CD95) on the target cell by FasL or TNF on the CTL side (15).

CTLs in the gut mucosa

An early study demonstrated that intraperitoneal immunization with an allogenic tumour induced specific CTLs in the gut lamina propria and epithelium at a time when very few cytotoxic lymphocytes could be detected in peripheral lymphoid organs (9).

It is now established that administration of certain enteric viruses into the gastrointestinal tract also results in early appearence of virus-specific CTLs in Peyer's patches (16–21). Further, reovirus infection also induces effector $CD8^+$ $\alpha\beta$ TCR^+ CTLs in the intestinal epithelium (20). Similarly, rotavirus infection induces increased pCTLs in GALT and their dissemination throughout the murine lymphoid system (17). These findings suggest that after enteric infection or immunization, antigen-induced CTLs are disseminated from Peyer's patches via the lymphatic drainage (21), and could serve as one of the sources of pCTL progenitors destined to the epithelial defense system. The potential of this system in immune clearance of enteric viruses has been documented in several systems. Thus, mice orally infected with *Toxoplasma gondii* develop intraepithelial $CD8^+$ $\alpha\beta$ TCR^+ CTLs which can transfer protection when infused into naive recipients (22). Further, effector CTLs protected against gastritis in a suckling mouse model of rotavirus infection (23). In a series of elegant studies designed to define the host determinants of rotavirus immunity, it was shown that adoptively transferred $CD8^+$ T cells mediated clearance of rotavirus infection in severe combined immunodeficiency (SCID) mice (24–26). Although S-IgA can also neutralize rotavirus infection of epithelial enterocytes (27), these studies indicate that $CD8^+$ CTLs are of central importance in rotavirus immunity.

Worth mentioning are the results of recent studies showing that intrarectal immunization of mice with a synthetic, multideterminant human immunodeficiency virus (HIV) peptide induced long-lasting, antigen-specific CTL memory in both the inductive (Peyer's patch) and effector (lamina propria) mucosal sites, and protected mice against infection via mucosal challenge with a recombinant vaccinia virus expressing HIV-1 gp160 (28). These studies provide evidence for the ability of

CTL in the mucosa to mediate protection against viral transmission.

CTLs in the airway mucosa

The kinetics of activated CTLs following virus clearance suggest that these cells do not play a very critical role in prevention of reinfection by respiratory viruses, such as respiratory syncytial virus (RSV), influenza and parainfluenza viruses. In contrast, studies in immunosuppressed animals indicate that CTLs play a central role in the resolution of established respiratory viral infections (29–31). It is likely that CTLs play also a major role in the clearance of established respiratory virus infections in humans. Studies in patients who had undergone immunosuppressive chemotherapy indicate that these patients suffer more frequent and severe infections with RSV, influenza, or parainfluenza viruses (32).

Detailed studies of immune responses after intranasal infection of rodents with influenza virus have revealed additional immune pathways involved in virus clearance. In this model, use of CD4 co-receptor knockouts or other mice in whom this subset had been depleted did not affect induction of pCTLs or alter significantly clearance of infection (33). In another study, clearance of influenza was not altered by the use of β_2 microglobulin knockout mice which lack $CD8^+$ T cells or mice which had been treated with monoclonal antibody (mAb) anti-CD8 (34). In the same model, the finding that γδ T cells with several Vδ chain specificities increase in the infected site as clearance occurs raises intriguing questions regarding the regulatory role of γδ T cells in antiviral immunity (35).

Several studies have also established that effector CTLs protect mice from RSV infection. The RSV F determinant, a 22 kD glycoprotein, is a major target of pCTLs and CTL induction by RSV or recombinant vaccinia virus expressing F glycoprotein-induced protective CTLs (36, 37). In a separate line of investigation, the murine RSV model was used to determine the relative importance of $CD4^+$ T cells, including T helper 1 (Th1)- and T helper 2 (Th2)-subsets which resulted in inflammation vs immunity. These studies clearly suggest that interferon-γ (IFN-γ)-producing $CD4^+$ Th1 cells as well as $CD8^+$ T cells are associated with recovery, while $CD4^+$ Th2-cells are not (38, 39). Interestingly, priming with inactivated RSV or F glycoprotein induced $CD4^+$ Th2 cells while live RSV elicited the Th1-type pathway (38, 39).

CTLs in the genital tract mucosa

The rhesus macaque model of vaginal infection with simian immunodeficiency virus (SIV) has been useful in studies of local immunity to SIV in the female reproductive tract (40). Recent studies in this model have provided direct evidence that pCTLs occur in female macaque reproductive tissues, and that infection with SIV induces effector CTL responses in the vaginal mucosa (41). Interestingly, macaques vaginally infected with an SIV/HIV-1 chimeric virus (SHIV) displayed gag-specific CTLs in peripheral blood and resisted two challenges with virulent SIV (42).

In a recent study involving HIV-1 infected women, cervical T-cell lines established from cytobrush specimens were shown to lyse autologous targets expressing HIV-1 proteins (43). Class II MHC-restricted $CD4^+$ CTL clones lysed targets expressing env gp41 or infected with HIV-1. Class I major histocompatibility complex (MHC)-restricted $CD8^+$ clones recognized HIV-1 Gag- or Pol-expressing targets. This study provided evidence for an MHC-restricted CTL effector function in the human female genital tract mucosa.

Regulatory T cells in the mucosal immune system

T cells are required for mucosal immunity, whether it develops as inflammation, or as tolerance or as help for specific S-IgA antibodies or CTLs in response to vaccines. B-cell commitment (μ→α switching) and B–T interactions which result in the induction of plasma cells producing polymeric IgA (pIgA) are of central importance to mucosal immunity. Cytokines and chemokines produced by $CD4^+$ and $CD8^+$ T-cell subsets and by classical APCs (e.g. dendritic cells (DCs, macrophages and B cells)) as well as by non-classical APCs (e.g. epithelial cells) contribute to all aspects of normal mucosal immunity, tolerance and inflammation in the immune response.

Regulatory T cells can be classified as: 1) naive, or those which have not yet encountered antigen; 2) activated (effector); and 3) memory types. Effector and memory T cells are both actively engaged in the immune response. The mucosal migration patterns of the three major subsets, along with the homing of B lymphocytes, form the cellular basis for the common mucosal immune system (44). Naive $CD4^+$ precursors of Th cells (pTh) normally recognize foreign peptide in association with MHC class II on APCs and express an αβ TCR^+, $CD3^+$, $CD4^+$, CD8 phenotype. On the other hand, precursor CTLs (pCTLs) express αβ TCR which usually recognize foreign peptide in the context of MHC class I on target cells and exhibit a phenotype of $CD3^+$, $CD4^-$, $CD8^+$. Thus, the MALT can be considered as a significant reservoir of pTh cells and pCTLs so that encounter with bacterial or viral pathogens can result in the induction of $CD4^+$ Th cell and $CD8^+$ CTL responses.

As $CD4^+$ Th cells differentiate in response to foreign antigens, they produce distinct cytokine arrays. Naive (pTh) cells first produce interleukin (IL)-2 and then develop into T cells

producing multiple cytokines (including both IFN-γ and IL-4), a stage often termed Th0 (45, 46). The tissue microenvironment (47), the cytokine milieu and the nature of the antigen influence the further differentiation of Th0 cells. Thus, infection with intracellular bacteria leads to the formation of Th1 cells and these cells often develop following production of IL-12 by activated macrophages (48, 49). Exogenous antigens in the mucosal microenvironment can trigger $CD4^+$, $NK1.1^+$ T cells to produce IL-4 for initiation of Th2-type responses (from Th0 cells) (50, 51). Th2-type cells also produce IL-4 for expansion of the Th2 subset as well as IL-5, IL-6, IL-9, IL-10, and IL-13 (52–56). This Th2 array may include production of IL-4; however, the reader should appreciate that individual cytokines are regulated through different signal transduction pathways so that all Th1 or Th2 cells do not produce the entire array. The Th2 array of cytokines is of importance for B-cell isotype switches, and for supporting IgG1, IgG2b, IgE, and IgA responses in the mouse system (54, 57, 58).

Th1 and Th2 cells are also sensitive to cross-regulation by each other. For example, IL-12 and IFN-γ produced by Th1 cells inhibits proliferation of Th2 cells, causes an isotype switch from IgM to IgG2a (50), and inhibits isotype switching induced by IL-4 (59). Conversely, Th2 cells regulate the effects of Th1 cells by secreting IL-10 which in turn inhibits Th1 cells, from secreting cytokines such as IL-12 and IFN-γ, thus decreasing IFN-γ-mediated inhibition of Th2 cells.

While the bulk of available evidence in mice suggests that mucosal tissues favor the development of Th2-type responses, the situation is not as clear-cut in humans. Thus, freshly isolated human lamina propria T cells contain an exceptionally high frequency of IFN-γ-producing cells (60, 61) but comparatively few cells producing IL-4, IL-5 or IL-10 (61). Furthermore, even in mice, infection with recombinant *Salmonella* leads to the formation of Th1 cells and Th2 cells in Peyer's patches (62). On the other hand, induction of IgA production to protein antigens is highly dependent on T helper cells (63–71). In humans, TGF-β and IL-10 in concert with IL-4 have been shown to promote B-cell differentiation into IgA-producing cells (64, 65). In this regard, it appears that in addition to resident T cells, which produce large amounts of IL-4, IL-10 and transforming growth factor (TGF)-β, human epithelial cells, also of intestinal origin, provide a major source of TGF-β and IL-10 (unpublished observations). This suggests that co-operation between neighboring lymphocytes and epithelial cells in the mucosal microenvironment is pivotal not only to promote the selective transport of newly formed IgA across epithelia but also for programing preferential maturation of IgA-committed B cells.

Finally, an additional type of $CD4^+$ regulatory T cell has been cloned from the mesenteric lymph nodes of rodents fed repeatedly with antigens (72). These cells or "Th3" cells, produce TGF-β, variable amounts of IL-4 and IL-10, but no IL-2 or IFN-γ, and appear to exert downregulatory properties on Th1 cells (73). Since TGF-β happens to serve as switch factor for murine and human IgA, and appears to play a major role in mucosal tolerance (reviewed in (74)), these cells may have an important function in mucosal homeostasis.

Mucosal inductive sites and generation of disseminated and compartmentalized secretory immune responses

Generation of an immune response at mucosal surfaces, where many significant infections begin, is not readily achieved by the conventional route of parenteral injection, although this is usually effective in eliciting circulating antibodies and systemic cell-mediated immune responses. In contrast, mucosal administration of antigens may result in the concomitant expression of antibody responses in various mucosal tissues and secretions, usually without a pronounced systemic immune response (85).

Extensive studies concerning the origin of B- and T-lineage lymphocytes that ultimately populate mucosal tissues and secretory glands, and of immunization routes effective in the induction of secretory antibody responses, indicated that the MALT can be divided into two functionally distinct compartments; namely, inductive and effector sites. This physiological division is of paramount importance in the design of vaccines effective in the induction of protective immunity within the mucosal immune system and, in particular, its humoral branch.

Experiments performed in animal models and more recently in SCID mice engrafted with human mucosal lymphoid cells revealed that the inductive sites present in certain locations, such as Peyer's patches in the small intestine, or in some species the tonsils in the upper aerodigestive tract, function as primary sources of precursor cells that migrate through the lymphatics and blood, and after directed extravasation populate remote mucosal tissues and glands (75–78). These studies have led to the notion of a common mucosal immune system (Fig. 1).

It is generally believed that antigens taken up by specialized epithelial cells ("M" cells or "membranous cells") covering mucosal inductive sites can be channeled to parenchymal macrophages, DCs, B lymphocytes, and even mast cells, and/or they can be processed and perhaps presented directly by epithelial cells to underlying B and T cells. Following interaction of the antigen with accessory cells and cognate helper T cells

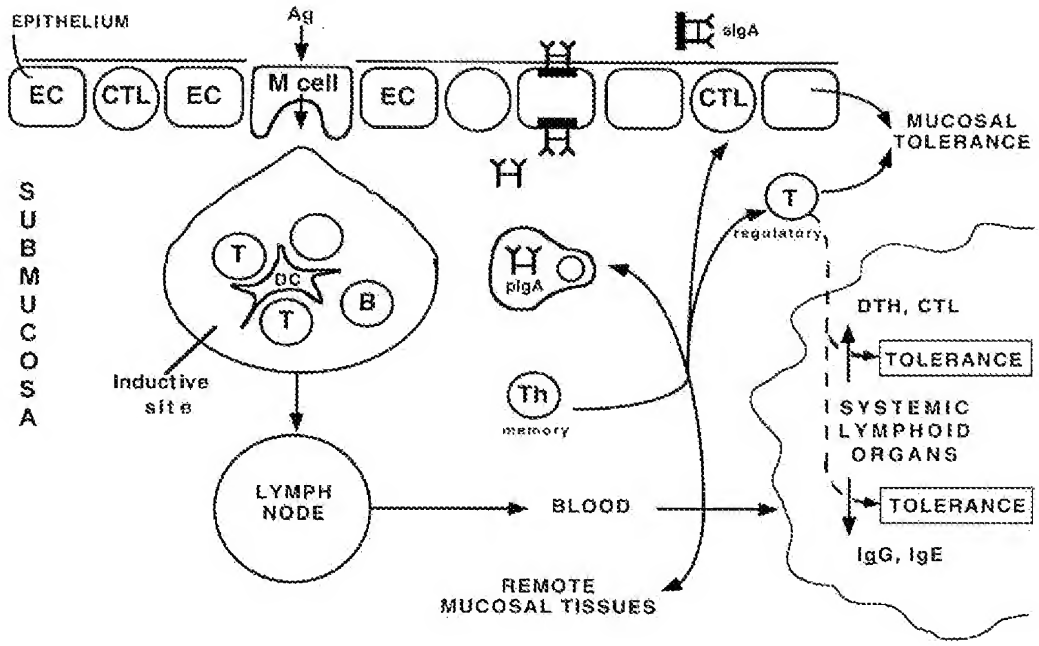

Fig. 1. Consequences of antigen uptake at mucosal surfaces. Uptake of antigen (Ag) through M cells or absorptive epithelial cells (EC) may result in induction of localized or disseminated mucosal and systemic immune responses and/or tolerance. CTL, cytolytic T lymphocytes; DC, dendritic cells; DTH, delayed-type hypersensitivity; pIgA, polymeric IgA; sIgA, secretory IgA.

and/or B lymphocytes in the local lymphoid microenvironment, an immune response may ensue. With the majority of antigens this results presumably in suppression of specific immunity – "mucosal tolerance" (see below). However, development of an active immune response may also follow mucosal intake of antigen, and the characteristics of the immune response generated, including the balance between active immunity and suppression/tolerance may be influenced by several factors, including the nature of the antigen, the type of accessory cells, cytokines and lymphocytes involved, and the genetic background of the host. Two major types of active immune responses may develop either concomitantly or separately: antibody formation (mainly SIgA) and cell-mediated immunity. The sensitized immunocytes, in particular antigen-sensitized B cells but presumably also T cells, leave the site of initial encounter with antigen, e.g. a Peyer's patch, transit through the thoracic duct, enter the circulation and then seed both that same mucosa and other distant yet privileged mucosal sites. In their new locations, the committed B cells may further differentiate into plasma cells producing antibodies, mainly dimeric IgA, under the influence of locally produced cytokines.

This IgA cell cycle was first elucidated in animals through the adoptive transfer of cells from the gut- and bronchus-associated lymphoid tissues (GALT and BALT) into recipients whose mucosal tissues and glands were populated by IgA plasma cells of donor origin. Most importantly for vaccine development, evidence for the existence of a common mucosal immune system in humans has been strengthened in recent years by several studies. In addition to the detection of specific S-IgA antibodies in remote secretions induced by natural exposure to antigens or oral immunization, analyses of IgA-secreting cells from peripheral blood and mucosal tissues after enteric immunization provided strong support for this concept (79–81). Differential utilization of organ-specific endothelial cell recognition mechanisms by circulating IgA immunoblasts induced after mucosal vaccination has also been demonstrated in humans (82, 83). Taken together with studies in the murine

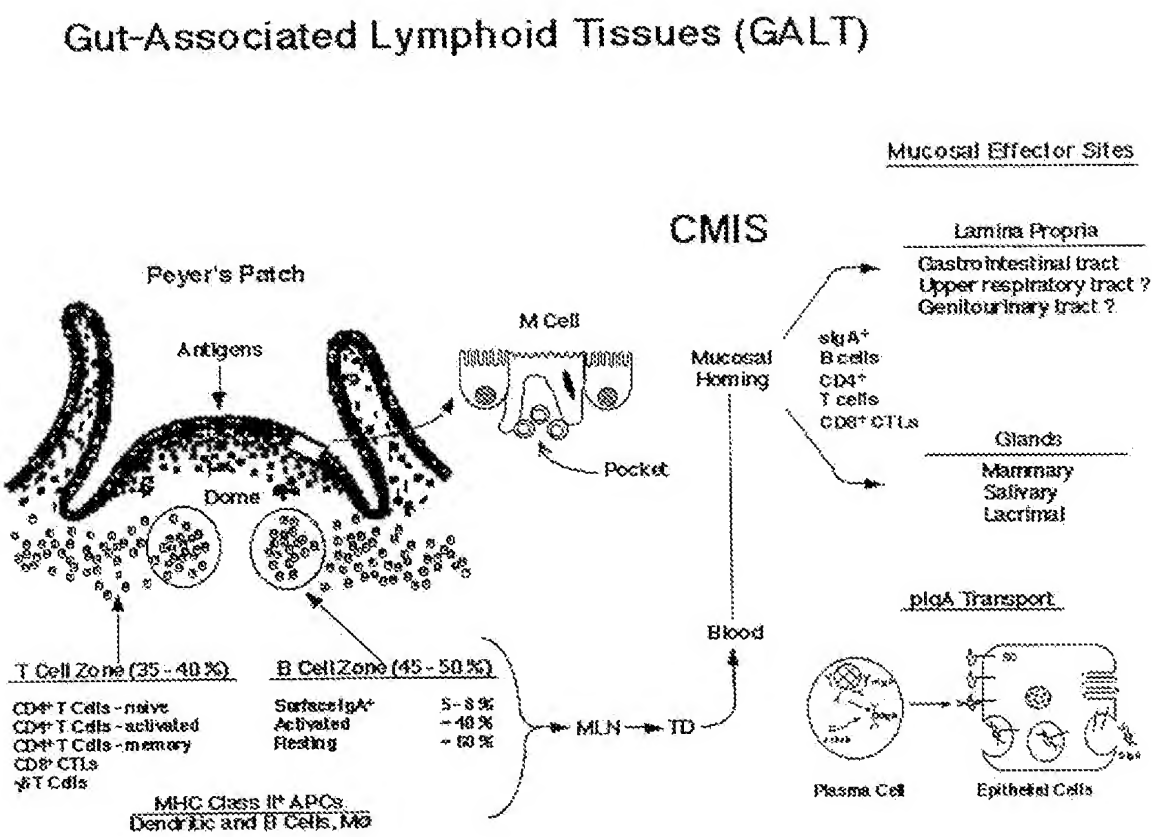

Fig. 2. Gut-associated lymphoid tissues (GALT) and the common mucosal immune system (CMIS). MLN, mesenterial lymph node; TD, thoracic duct; M cell, membranous cell; pIgA, polymeric IgA.

system, this finding may explain both the unification of humoral immune responses in diverse mucosal sites and the physiologic segregation of mucosal from non-mucosal immune mechanisms.

Based on the concept of a common mucosal immune system through which a fraction of lymphocytes activated in the gut, e.g. by ingestion of antigen, can disseminate immunity not only in the intestine but also to other mucosal and glandular tissues, there is currently much interest in the possibility of developing oral vaccines against both enteric and non-enteric mucosal infections, e.g. in the respiratory or genital tract. However, recent studies involving immunizations of human and non-human primates as well as rodents with prototype non-replicating immunogens, such as cholera toxoids or cholera toxin indicate that a substantial degree of subcompartmentalization exists within the mucosal immune system, and even within a given mucosal organ such as the gut, regarding both homing of IgA plasma cell precursors and final redistribution of their progenitors.

The MALT is comprised of anatomically defined lymphoid microcompartments, such as the Peyer's patches in the small intestine, the appendix and solitary follicles in the large intestine and in the rectum, the nasal mucosa and the tonsils at the entrance of the aerodigestive tract, which serve as the principal mucosal inductive sites where immune responses are being initiated (84–87). It also contains diffuse accumulations of large numbers of lymphoid cells that do not associate into apparently organized structures. These cells are either distributed in the lamina propria or interspersed among epithelial cells in mucosal tissues and glands, and form the mucosal effector sites where immune responses are being expressed. The gut mucosa is particularly well invested with such diffuse lymphoid tissues.

More important for vaccine development is the fact that immunization at certain inductive sites may give rise to a humoral immune response preferentially manifested at certain effector sites. Thus, a given inductive site may serve as a preferential but not exclusive source of precursor cells for certain mucosal tissues.

Inductive sites in the gut-associated lymphoid tissues (GALT)

The gut contains the most abundant lymphoid tissues and includes organized as well as diffuse lymphoid elements. Organized lymphoid tissues are comprised of two units – B-cell follicles and para- or interfollicular T-cell areas – assembled within a matrix of loose connective tissue and follicular DCs. These follicles occur singly or in groups and harbour variable numbers of macrophages and T cells (88).

Follicle-dome structures form the main lymphoid component of the Peyer's patches in the jejunum and ileum and are also found in the large intestine, and especially in the appendix. These structures appear to play an important role in the induction of disseminated immune responses to oral vaccines (Fig. 2). Typically, the follicles contain a majority of B cells, approximately half of which are activated. The T-cell zone comprises a majority of $CD4^+$ T cells; $CD8^+$ $\alpha\beta$ TCR T cells are mainly located in the parafollicular area whereas $CD8^+$ $\gamma\delta$ TCR T cells are rare. The dome is covered by a specialized epithelium or "follicle-associated epithelium" (FAE), containing antigen-transporting M cells.

The cecal and colonic mucosae are also variably invested with comparable lymphoid patches, although the epithelium covering these lymphoid aggregates does not show the specializations of M-cell-containing FAEs (89, 90).

Clusters of follicles are also found adjacent to the ano-rectal junction (91, 92). The potential importance of the rectal lymphoid tissues as an IgA inductive site and as a source of IgA plasma cell precursors is suggested by several studies. First, the predominance of IgA2 cells over IgA1 cells in the lamina propria of the large intestine clearly diverges from the relative apportioning of the two in other mucosal tissues, such as in the small intestine and in the upper large intestine (93). Further, rectal immunization of humans, non-human primates and rodents has been shown to induce strong secretory antibody responses in the rectal mucosa. Although in most instances, rectal immunization induced secretory antibody responses in the rectal mucosa itself (94–100), in some instances, rectal vaccination could induce specific antibodies in serum, and also in secretions from remote mucosal organs, such as saliva (102), and genital secretions (92–94, 96). Although the potential of the rectal mucosa to serve as site of induction of mucosal responses is now well established, it should be pointed out that rectal immunization of macaques with CT, one of the most powerful mucosal immunogens, was shown to be poorly effective at inducing an immune response in the upper part of the intestine and especially in the small intestine (100).

Recently, a new type of "organized" lymphoid tissue, termed the cryptopatch, has been identified in the murine intestine (101). These consist of microscopic aggregates (a few hundred cells) of immature lymphocytes clustered in the lamina propria. Although cryptopatches have not yet been identified in humans, it has been proposed that they represent a precursor source of intestinal intraepithelial T cells. The potential role of these structures in the induction of active immunity or tolerance to mucosally encountered antigens remains to be explored.

Inductive sites in the naso-pharyngeal lymphoreticular tissues (NALT)

Several recent studies have emphasized the importance of the nasal cavity for the generation of mucosal and systemic immune responses that may exceed in magnitude those induced by oral immunization (83, 102–111). When introduced into the nasal cavity, usually along with mucosal adjuvants, viral and bacterial antigens induce superior immune responses in external secretions such as saliva and, surprisingly, in female genital tract secretions. Because neither IgG nor IgA antibody titers in vaginal washes correlate with serum antibody responses, it is assumed that antibodies of both isotypes are predominantly of mucosal origin. This finding may have important implications for the design of vaccines effective in the induction of immune responses in the genital tract.

Thus, different immunization routes (intranasal and oral) can induce generalized mucosal immune responses, although the relative representation of dominant antibody isotypes may vary. Nevertheless, nasal immunization appears to induce S-IgA immunity in a broader range of mucosal tissues than oral vaccination. This may be explained by the recent observation that circulating IgA-secreting cells induced after nasal vaccination express a more promiscuous profile of homing receptors than their corresponding counterparts raised after oral or rectal immunization (82, 83). Whether such antibody responses are also induced in the male genital tract remains to be determined.

Immunohistochemical studies have indicated that the epithelium of the human nasal mucosa contains intraepithelial lymphocytes (IELs) staining predominantly for CD3, CD2, CD8, and CD5, but lacking Leu8 (homing receptor analogue of Mel14) (123, 124). Few (<10%) nasal IELs stain for CD4. Nasal IELs rarely express HML1, whereas the majority of intestinal, tonsillar and adenoidal IELs are $HML1^+$. $HLA\text{-}DR^+$ intraepithelial DCs cells have also been identified in nasal mucosa specimens. Virtually no B cells and no organized subepithelial lymphoid tissue are found in the normal human nasal mucosa. In contrast, the nasal mucosa of adult rats and mice comprise lymphoid structures with distinct T- and B-cell areas (112, 113). Furthermore, the majority of intraepithelial lym-

phocytes populating the nasal epithelium are sIg$^+$ B cells with a few CD4$^+$ or CD8$^+$ T cells in rats (112), whereas the murine nasal epithelium is infiltrated mainly with CD4$^+$ T lymphocytes with very few B cells (113). Thus, the human nasal mucosa seems to differ from that of rodents – the most commonly used animals in preclinical vaccine studies – and also from endodermally derived human mucosae, such as the tonsils and adenoids. The latter lymphoid structures appear to be the major sites of induction of mucosal immune responses to inhaled antigens in humans.

Strategically positioned at the entry of the aerodigestive tract, the palatine, lingual and nasopharyngeal tonsils (Waldeyer's ring) are continuously exposed to ingested and inhaled antigens. These glands possess structural features resembling both lymph nodes and Peyer's patches (87), including a lymphoepithelium which contains M cells in tonsillar crypts, and which are essential for antigen uptake (Fig. 2). In addition, germinal centers containing B and T cells, plasma cells, and professional APCs are also present. Several observations have suggested that these lymphoid tissues may serve as a source of precursors of IgA plasma cells found in the upper aerodigestive tracts: the distribution of IgA1- and IgA2-producing cells in the nasal and gastric mucosae and in lacrymal and salivary glands is similar to that found in tonsils. Furthermore, the S-IgA immune response to oral poliovirus vaccine is reduced in tonsillectomized children compared to children with intact tonsils (114). Direct unilateral injection of antigens into the tonsil of human volunteers resulted in the induction of local immune responses manifested by the appearance of antigen-specific IgG-producing and to a lesser degree IgA-producing cells in the injected tonsil (103). Furthermore, considerable numbers of antibody-forming cells with a similar distribution of isotypes were detected in peripheral blood, suggesting that the tonsils may serve as an inductive site, analogous to Peyer's patches, that is effective in the stimulation of generalized mucosal immune responses.

Inductive sites in the genital tract mucosa

Although long considered as immunologically incapable of supporting an active immune response against locally encountered antigens, but especially against spermatozoa or preimplantation embryo, the female reproductive tract mucosa has been shown to comprise all cell populations required for initiating an immune response. HLA-DR$^+$ Langerhans cells have been identified in the vaginal and cervical epithelia (117, 118), being most abundant in the vulval epithelium. Intraepithelial T cells have been identified at all sites and comprise a majority of CD8$^+$ cells, the latter being particularly abundant in the transformation zone (117). A significant proportion of these cells express perforin and TIA-1 (119), suggestive of cytolytic capacity. In contrast, CD4$^+$ T cells are rarely found in these epithelia but predominate in the submucosa of the vagina, cervix and fallopian tubes. The submucosa of the vagina, cervix and fallopian tubes contains large numbers of J-chain- and IgA1- and IgA2-containing plasma cells, and the epithelium of the fallopian tubes and cervix stains for secretory component and has thus the potential to transport polymeric IgA (120, 121).

Vaginal immunization of human and non-human primates with cholera toxoid and CT, respectively, has been shown to evoke serum antibody responses and secretory IgA and IgG responses in cervico-vaginal washes (100, 122, 123). Further, in female macaques immunized with CT applied into the vagina, these responses were associated with large numbers of IgG and IgA antibody-secreting cells (ASCs) in the cervical and vaginal mucosae (100), demonstrating active production of such antibodies at local sites. However, this route of immunization was poorly if at all effective in inducing an IgA immune responses in other mucosal compartments (100).

Although all components of the mucosal immune system are present in the female reproductive tract, the precise sites of induction of secretory immune responses in this organ are largely unknown. The most likely scenario involves antigen uptake by intraepithelial Langerhans cells which then migrate to draining lymphoid tissues where they actively educate naive T and B cells. These in turn migrate via the efferent lymph and the blood circulation and seed to the submucosa where they differentiate into effector cells. Draining iliac lymph nodes appear to be the most likely site of initiation of such responses (92).

Thus, within the context of the common mucosal immune system, certain organs may favor the development of IgA-committed precursors B cells whose progeny is preferentially destined to particular mucosal locations. This new knowledge should call for more site-directed vaccination strategies.

Mucosal anti-infectious vaccines

Since Jenner introduced vaccination over 200 years ago, fewer than 50 vaccines have been approved for human use, nearly half of which are improved versions of earlier forms. All but five of the current vaccines – the oral poliovaccine, the oral killed whole cell/B subunit cholera vaccine, the oral *Salmonella typhi* vaccine, an oral rotavirus vaccine and nasal live-attenuated influenza virus vaccines which have completed phase III clinical testing – are administered parenterally and as such do not provide significant mucosal immunity.

It is now almost axiomatic that in order to be efficacious, vaccines against mucosal infections must stimulate the MALT, and that this goal is usually better achieved by administering immunogens by a mucosal route rather than parenterally. However, even so, stimulation of secretory immune responses by mucosal administration of most non-replicating antigens is often relatively inefficient, requiring multiple administrations of large quantities of immunogens and yielding most often tolerance and, if at all, modest immune responses. This conclusion is physiologically justifiable since the primary function of the mucosal immune system is to prevent the overstimulation of the entire immune system. It does so by eliminating mucosally applied antigens with denaturing acids, degradative enzymes and other innate factors as well as through intestinal peristalsis and ciliary movement on epithelia in the respiratory tract.

Against this background, mucosal vaccinologists have elaborated numerous delivery systems and adjuvants which partly circumvent such natural obstacles. The ever-growing list of mucosal delivery systems described in the literature bears witness to the difficulties encountered and to the limited practical usefulness of most delivery devices. We will therefore concentrate on the most promising systems. Furthermore, it should be emphasized that most of the results have been obtained in animal models rather than in humans with all unavoidable limitations.

Inert vaccine delivery systems

Because antigens are more immunogenic in particulate form than in solutions, and because they are vulnerable to antigen-degrading enzymes and acids, they have often been incorporated into vehicles that are by themselves non-toxic and non-immunogenic, and which protect vaccine material from degradation, enhance their uptake from mucosal surfaces, and may exhibit an adjuvant effect. Gelatin capsules coated with substances (e.g. cellulose acetate phthalate) that dissolve at alkaline pH in the intestine but not at acid pH in the stomach have been used for oral delivery of bacterial and viral vaccines in several studies performed in humans but their usefulness has never been systematically assessed (125–127).

To avoid the fast passage of free antigens, mucoadhesive polymers that adhere to mucosal surfaces and thus extend the time of exposure of vaccines have been used in several recent studies (123, 128, 129). When compounds such as highly viscous inert polysaccharide eldexomer (123) and carboxymethyl cellulose (128) are used with an antigen such as influenza virus or CT-B that has been orally, intranasally, or intravaginally administered, both local mucosal and systemic immune responses are induced. Other compounds that have been considered are carbapol, polycarbophil, sodium alginate, and hydroxypropyl cellulose, which are used in medicine for drug delivery.

Liposomes have been used for mucosal vaccination in a few studies, while bacterial or viral antigens have been administered orally or intranasally to rodents, monkeys and humans, resulting in the induction of mucosal and systemic immune responses (reviewed in (130)). A related antigen delivery system, cochleates, composed of protein–phospholipid–calcium precipitates with entrapped antigens, has been used for mucosal delivery of glycoproteins or peptides from influenza virus and SIV (131). As with liposomes, humoral as well as cell-mediated immunity (CMI) have been induced when given by the oral route.

Biodegradable microspheres composed of antigens incorporated into polymers of lactic and glycolic acid have been used far more extensively in mucosal vaccinology than any other inert delivery system (132–135). Microspheres have gained popularity because of their stability which allows them to protect incorporated antigens from acids and enzymes, and because of the ease with which their size and rate of biodegradation can be modified. Further, microspheres are by themselves non-antigenic and so can be easily reused, offering an advantage over live vectors. The rate of release of antigens can theoretically be controlled by mixing fast- and slow-releasing microspheres, allowing for the induction of combined primary and secondary immune responses by a single immunization (132). Finally, it would seem possible to incorporate immunoregulatory cytokines or DNA encoding for different antigens to achieve a desired immune response, and attempts to do so are in progress.

These obvious attractive features of the inert delivery systems are counterbalanced by serious disadvantages. Specifically, the disappointingly low absorption rate, usually much less than 1% of the ingested dose, renders the procedure expensive. Moreover, the use of organic solvents during the incorporation of antigens may severely compromise the immunogenicity of such vaccines. However, attempts are being made to increase the absorption rate and to develop conditions of incorporation that minimize degradation of antigens.

Mucosal lectin-like molecules as carrier-delivery systems

Oral administration of small amounts of protein antigens covalently coupled to carrier molecules with known affinity for mucosal epithelial cells, such as CT, *Escherichia coli* heat-labile toxin (LT), CT-B and LT-B, can elicit mucosal and also, under certain conditions, systemic antibody responses to the conju-

gated antigen (136–138). This strategy has also recently been utilized to induce mucosal immune responses to carbohydrate antigens (139).

Fusion proteins created from hybrids of CT-B or LT-B with genes encoding heterologous antigens have also been generated (140, 141), and are preferable to chemically coupled proteins in that they are free of contaminating holotoxin and are uniform in structure. Overexpression systems have also been developed to facilitate large-scale production of such fusion proteins (142). Others have created fusions between antigens and CT-B-CT-A2 mutants which lack the toxic A1 subunit (143–145). Interestingly, though oral administration induced serum IgG antibodies, maximum mucosal IgA responses required addition of the whole CT. Thus, it appears that CT-B is an excellent carrier molecule due to its ability to bind to GM1 receptors on epithelial cells but at best exhibit low adjuvant properties.

Live recombinant microorganisms as mucosal delivery syterns

It has long been assumed that only live vaccines would efficiently stimulate a mucosal immune response since the majority of microbial pathogens colonize or enter through mucosal surfaces and hence have evolved strategies to circumvent natural physico-chemical barriers. Furthermore natural infection with a number of microbial pathogens induce strong immune responses in both mucosal and systemic compartments and these responses are often protective against reinfection. The use of live attenuated recombinant bacteria and viruses, which can be genetically engineered to synthesize unrelated antigens, has the obvious advantage that it is theoretically possible to package the same recombinant organism with genes encoding several unrelated antigens. Recombinant bacterial and viral vectors which contain genes from unrelated pathogens that encode important virulence factors have been explored in many experimental vaccine studies (146). The development of recombinant vectors has been facilitated by rapid progresses relating to complementary DNA cloning of large DNA inserts and the advancement of polymerase chain reaction-based techniques. The ability of a vector to colonize specific mucosal locales and so to generate an immune response at desired sites is an important factor in determining its suitability as vaccine vehicle. The most important factor to be considered is the ability of a given vector to induce certain types of immune responses. Experience so far indicates that in general, mucosal administration of recombinant bacteria or viruses may induce mucosal S-IgA responses, and in most instances these responses are associated with priming of Th1-type helper responses which may not always be desirable. Although many bacterial and viral vectors have been used in animal models (147), only recombinant *Salmonella*, adenoviruses and poxviruses have been used with limited success in humans.

Salmonella presented by the oral route replicates in Peyer's patches and disseminates via the mesenteric lymph nodes to systemic sites, such as the spleen. This characteristic pattern of migration allows *Salmonella* to induce a broad-based immune response which includes cell-mediated as well as serum and mucosal antibody responses. Attenuated avirulent *Salmonella* strains have therefore received particular attention as mucosal vaccine delivery vectors for recombinant proteins associated with virulence (148–153). Although a large number of genes from bacteria, viruses, and parasites have been expressed in attenuated *Salmonella*, a critical balance between attenuation, adequate expression and immunogenicity has often been difficult to achieve.

Only recently have studies begun to characterize both mucosal T- and B-cell responses to recombinant antigens expressed in *Salmonella*, particularly in terms of the balance between antigen-specific CD4+ Th1 and Th2 cells and their influence on the types of ensuing immune responses. Mice given an oral attenuated *Salmonella* vaccine displayed CD4+ Th cells which produced IFN-γ and IL-2, but not IL-4 (154), immediately raising the question of as to how such T-cell help is also provided for mucosal S-IgA antibody responses. Oral delivery of rSalmonella which express the *Tox* C gene of tetanus toxoid (TT) resulted in predominant serum IgG2a and mucosal S-IgA antibody responses (62, 155). Splenic and Peyer's patch T cells selectively produced IFN-γ and IL-2 as well as the Th2-type cytokine IL-10. IL-4 was shown not to be involved in anti-*Salmonella* and anti-TT IgA responses by experiments in IL-4 knockout mice. Interestingly, CD4+ Th cells in these IL-4 knockout mice exhibited two distinct cytokine patterns: a Th1-phenotype of IFN-γ and IL-2, as well as T cells which produced IL-6 and IL-10, but no IL-5 (155).

Intranasal immunization has emerged as perhaps the most effective route for induction of both peripheral and mucosal immunity to vaccines. The usefulness of *Mycobacterium bovis* strain Bacille Calmette Guérin (BCG) as a delivery system for recombinant antigens has been documented in several models. Specifically, BCG-vectored outer surface protein and pneumococcal protein A conferred protection against *Borrelia* and *S. pneumoniae*, respectively, when given nasally to mice (reviewed in (156)). Recently, attenuated *Bordetella pertussis* strains have been constructed by deleting the pertussis toxin gene and have been used as live vectors for intranasal delivery of heterologous antigens expressed at the surface of *B. pertussis* via fusion to the filamentous hemagglutinin (*FHA*) gene (157). Intranasal delivery

of *B. pertussis* expressing a parasite antigen fused to FHA induced strong mucosal immune response but relatively modest serum antibody responses to the fused antigen. Nevertheless, such an approach could be used for the construction of combined vaccines to protect not only against whooping cough but also against other respiratory infections.

Adenoviruses (r-Ad) have proven to be rather attractive systems for tissue targeted delivery of mammalian and microbial genes in gene therapy and in vaccinology, respectively. However, in both instances host immune responses develop to r-Ad and precludes readministration of the vector (158, 159). Despite these drawbacks, the use of r-Ad remains a promising approach in both arenas. Most studies to date have been performed with r-Ad from which the *E1* and *E3* genes have been deleted, rendering virus replication deficient. A major advantage of the current generation of r-Ad vectors is their capacity to accommodate large amounts of exogenous cDNA (7–8 kilobases), a feature which has been used to express virulence genes from a number of viral pathogens. However, the virions retain full competence for infection of mucosal epithelial cells in the respiratory tract (159, 160). In recent studies, r-Ad expressing herpes simplex virus glycoprotein B was shown to be a most effective vaccine when given by the intranasal route to mice (104, 105, 161). This regimen induced serum IgG as well as pulmonary IgA anti-glycoprotein B responses which correlated with protection from challenge. Further, this vaccine induced an effective CTL response (162). The same authors have also demonstrated that this vector induced specific secretory immune responses in the rodent female genital tract (104). This mucosal route of immunization also appears to be safe and effective, since intranasal, intratracheal and oral delivery of SIVenv glycoprotein by an r-Ad to macaques (163) and intranasal immunization of chimpanzees with a r-Ad expressing HIV *envelope* and *gag* genes (102) resulted in significant serum antibody responses. These antibody responses were broad-based, and when boosted with a gp160 subunit vaccine, elicited serum neutralizing antibodies. Recombinant Ad have also been used as vector for rabies virus in order to orally vaccinate foxes (164). In addition, oral administration of r-Ad expressing RSV and hepatitis B virus sequences have successfully induced circulating antibody responses in dogs and chimpanzees (165, 166). Moreover, intranasal or enteric immunization with a r-Ad expressing bovine coronavirus hemagglutinin-estrase glycoprotein induced virus specific mucosal immune responses in cotton rats (167). Despite these significant advances, further studies will obviously be required to determine optimal mucosal immunization protocols and to enhance the expression of desired antigens while diminishing responses to the vector to allow for vaccine readministration. In this respect, a recent study indicates that induction of mucosal tolerance (see below) to the r-Ad vector can be accomplished by prior mucosal exposure to inactivated adenoviral vector (168).

Recombinant poxviruses have been used in numerous studies including human clinical trials when given by systemic routes (by scarification, subcutaneously or intramuscularly), but studies documenting their mucosal immunogenicity are still limited. This is somewhat surprising given the effectiveness in wild animals of an oral vaccine against rabies (169) based on a recombinant vaccinia virus expressing the rabies glycoprotein gene (170). Oral administration of recombinant vaccinia virus encoding influenza virus hemagglutinin and neuraminidase induced both systemic and secretory anti-influenza antibody responses in mice (171, 172). Although relatively straightforward to generate in high yields and to purify, replication-competent recombinant vaccinia viruses pose potential problems for being used as mucosal vectors, including the risk of widespread infection in immunocompromised individuals. Vector systems based on avian poxviruses (fowlpox or canarypox-ALVAC) or an attenuated vaccinia virus (NYVAC), have now been developed which allow only single round infections in mammalian cells (173). Intranasal immunization of ferrets with NYVAC and ALVAC vectors expressing canine distemper virus HA and F genes were equally potent in protecting these animals against CDV challenge; intraduodenal administration was only partly effective (174). Parallel evaluation of different vector systems is rarely found in the litterature. In one study, the efficacy of r-Ad and NYVAC vectors expressing pseudorabies gD glycoprotein was compared after intranasal and intramuscular immunization. They have demonstrated that, while the adenovirus proved more efficient than the poxvirus vector to elicit an antibody response, the survival times of animals after challenge with pseudorabies virus was comparable (175). More recently, the modified vaccinia Ankara (MVA) strain of vaccinia virus has attracted the interest of many investigators. Indeed, MVA is a highly attenuated non-propagative strain of vaccinia which has been obtained after more than 500 consecutive passages in chicken embryo fibroblasts (176). The immunogenicity of recombinant MVA-based vectors by the parenteral route has recently been demonstrated (177) and MVA holds promises as a safe and effective mucosal vaccine delivery system. More generally, such non-replicative poxviral vectors are promising mucosal delivery systems.

The use of RNA viruses as vectors initially lagged behind the use of DNA viruses, mainly due to the relative ease of manipulating DNA genomes. Given the progress in recombinant DNA technology, the development of RNA viruses as vac-

cine vectors has now caught up with DNA viruses (reviewed in (178)). Recombinant vaccine strategies using RNA viruses such as poliovirus, alphaviruses (Venezuelan equine encephalitis virus (VEE), Semliki Forest virus), and paramyxoviruses (RSV) as vectors are being actively explored for mucosal immunization. In particular, replication-competent VEE expressing HIV p18 and p24 gag (179) or influenza virus HA genes (180) have been shown to induce mucosal and cellular immune responses. Interestingly, a replicon vaccine vector system was developed from an attenuated strain of VEE (181). The replicon RNA consists of the cis-acting 5' and 3' ends of the VEE genome, the complete non-structural protein gene region, and the subgenomic 26S promoter. Immunization of mice with the VEE replicon expressing the influenza HA or Lassa virus N gene induced antibody responses against the expressed protein and demonstrated a capability for sequential immunization to multiple pathogens in the same host (181). Likewise, recombinant poliovirus can be engineered as replication-competent vector (182) or as replicon (183) (reviewed in (178)). The unique aspects of these viruses, including their port of entry, point to promising developments in mucosal vaccinology.

Mucosal DNA vaccines

Since Wolff and colleagues (184) reported that direct intramuscular injection of mice with plasmid DNA encoding the complete sequence of a gene resulted in durable expression of the encoded protein, immunization with plasmid DNA encoding virulence genes of several important pathogens has been shown to induce protective immunity in several preclinical studies (185). Although many issues, including safety considerations, will need to be carefully assessed, this approach offers substantial advantages. Aside from stability and ease of production, these include the induction of immunity by a non-infectious agent, and thus the possibility of vaccinating against organisms with a propensity for long-term persistence in the body, e.g. cytomegalovirus and HIV. Furthermore, DNA immunization would allow the selection of nucleic acids corresponding to a broad range of polypeptides to overcome the problem of MHC genetic restriction in outbred populations. In addition, the plasmid backbone can also be equipped to harbour sequences encoding cytokine genes so as to facilitate the type of immune response desired.

In principle, the DNA used for vaccination is a plasmid containing a bacterial origin of replication and an antibiotic resistance gene, required for selective plasmid production in *E. coli*. The gene of interest is cloned under the control of a strong promoter (usually a viral promoter). In addition to the expression cassette, the plasmid may contain immunostimulatory sequences which appear to be critical in governing the immunogenicity of the expressed gene. These sequences include a non-methylated CpG motif within a palindromic hexamer oligonucleotide sequence (186, 187) and have been reported to induce B-cell proliferation (188) and Th1 cytokine production (189, 190). The stimulatory properties of such motifs appears to be mediated via the selective activation of DCs (191). It is interesting to note that mucosal administration of synthetic oligodeoxynucleotides containing CpG motifs enhances virus specific responses, suggesting that such nucleotides could be used also as mucosal adjuvant (192).

Although the majority of studies so far have dealt with intracutaneous and intramuscular administration of DNA vaccines, this approach has gained popularity among mucosal vaccinologists. Naked plasmid DNA can be directly used for mucosal immunization (193–197). Thus, intranasal or intratracheal administration of a DNA plasmid encoding influenza virus hemagglutinin induced protection against a lethal influenza virus challenge in mice (185). More recently, intranasal immunization of mice with a DNA plasmid encoding lacZ and herpes simplex virus type 1 glycoprotein B (gB) induced lacZ and gB expression in lungs and cervical lymph nodes (195). Although a distal mucosal IgA response and an anti-HSV cell-mediated immune response were observed following three i.n. administrations of gB DNA, protection against lethal vaginal HSV challenge was lower than that seen after intramuscular administration of the same plasmid. Intrabuccal injection or nasal administration of plasmid DNA encoding measles virus hemagglutinin induced systemic MHC class I-restricted CTL responses (196). Further, intravaginal instillation of a plasmid DNA encoding the HIV-1 envelope glycoprotein elicits production of IgA and IgG antibodies in vaginal fluids and serum neutralizing antibodies (194). The same construct was employed to demonstrate that plasmid DNA vaccination of infant chimpanzees was well tolerated and could induce serum antibodies to HIV-1 (197).

As for conventional vaccines, several formulations have been developed to facilitate the uptake of plasmid DNA-based vaccines and to protect them from degradation in the mucosal microenvironment. The use of liposomes, cationic lipids, monophosphoryl lipid A for mucosal delivery of DNA (198–201) has been shown to increase both expression and immunogenicity of the corresponding protein, suggesting that the lipid matrix may also provide a secondary role as adjuvant by facilitating DNA uptake by APCs or by promoting local inflammation. In the case of oral delivery, plasmid DNA can also be protected from degradation in the gut by encapsulation

in poly(DL-lactide-co-glycolide) (PGL) microparticles (202, 203).

Attenuated intracellular bacteria such as *Salmonella*, which are retained within vacuoles in the infected cell, have recently shown promise for the delivery of plasmid DNA. Thus, infection of peritoneal macrophages with *S. typhi* carrying a plasmid encoding lac Z results in the transfer of plasmid DNA into the infected cell (204). Very recently, this approach has been employed to demonstrate that oral administration of *S. typhi* carrying a tumor antigen results in gene transfer in host DCs and protects mice against fibrosarcoma (205).

The mechanisms underlying the induction of an immune response after mucosal DNA vaccination are still unknown, but are likely to be analogous to those involved after systemic (intramuscular, intradermal) DNA administration (206–209): transfer of antigen from tissue-transfected epithelial cells to professional mucosal APCs (cross-priming), or direct transfection of professional APCs and especially DCs.

Edible vaccines

Mucosal vaccines would be more widely used – especially in developing countries – if they could be produced at lower cost, and distributed without refrigeration. The concept of vaccine production in transgenic plants was introduced recently (210), and was based on the premise that plants have the capacity to produce abundant biomass, and that recombinant immunogens could be produced in plant tissues. Since plants can be engineered to contain multiple foreign genes, multicomponent transgenic plant vaccines should therefore be feasible. Several plant expression systems, e.g. potato and tobacco plants, have been developed and shown to allow expression of microbial antigens such as hepatitis B surface antigen, *E. coli* LT-B, and even Norwalk Virus-like particles (VLPs) (reviewed in (211)). Although the levels of expression of recombinant antigens have so far been very disappointing, feeding animals with potato tubers expressing Norwalk VLPs or *E. coli* LT-B induced specific secretory IgA antibody responses and serum IgG responses to the transgenic protein (211). Developing strategies to increase antigen expression in edible plants, including the use of strong tissue-specific promoters remain one important challenge in this area of vaccine development.

Dendritic cells as vaccines against mucosal infections

DCs are potent APCs that play a central role in the induction of immunity as initiators and immunomodulators of immune responses (212). Owing to their capacity to prime T cells, DCs loaded *ex vivo* with tumor antigens have been extensively used as potential cancer vaccines (213, 214). Given promising preclinical results, studies are now underway in patients. That adoptive transfer of DCs pulsed *ex vivo* with infectious microorganisms could constitute a way to induce broad-based immunity against infections diseases has also been documented (215–217).

To date, the potential of DCs as vaccine carriers for the induction of mucosal responses is largely unknown. In this respect, a recent study has shown that DCs pulsed *ex vivo* with killed chlamydiae and subsequently transferred into naive recipients were able to induce protective $CD4^+$ Th1 immune responses against genital challenge with chlamydia (218). Although this study used bone marrow-derived DCs, it is the first one to document the efficacy of this approach against a mucosal infection.

Bacterial enterotoxins as mucosal adjuvants

The introduction of proteins including vaccines into mucosal inductive sites is an effective way to induce systemic unresponsiveness (mucosal tolerance). Thus, mucosal adjuvants are required not only to boost mucosal and systemic immunity, but also to prevent the induction of mucosal tolerance.

Although a variety of compounds have been reported to display adjuvant properties on mucosally co-administered antigens, the most powerful and hence most studied mucosal adjuvants are the *Vibrio cholerae* exotoxin CT, and its structural and biological analog LT. These macromolecules are composed of two structurally, functionally and immunologically separate A and B subunits (219). The B subunit of both toxins consists of five identical monomers, but the pentameric B subunit of CT (CT-B) binds only to GM1 ganglioside, while the B subunit pentamer of LT (LT-B) is more promiscuous and binds to GM1 as well as to asialo GM1, GM2, and glycoprotein receptors (220). After the B subunit binds to epithelial cell GM1 or GM2 receptors, the A subunit reaches the cytosol and binds to NAD to catalyze ADP-ribosylation of $G_{s\alpha}$. This GTP-binding protein activates adenyl cyclase with subsequent elevation of cAMP, which in epithelial cells results in secretion of water and chloride ions into the small intestine.

Mucosal exposure to CT and LT, which are both immunogenic, results in S-IgA and serum IgG antibodies, which are almost entirely restricted to CT-B or LT-B. More importantly, both toxins are potent mucosal adjuvants for unrelated proteins co-administered by oral, intranasal or even parenteral routes (221–223).

Recent studies suggest that oral or nasal immunization with proteins along with CT as adjuvant induces Th2-type responses in the intestinal mucosa (224–226). Such an immu-

nization protocol induced CD4+ Th2 cells in Peyer's patches and the spleen (224) as well as high IgG1 and IgE responses, in the absence of detectable IgG2a titers (226, 227). Further, oral immunization with keyhole limpet hemocyanin (KLH) and CT mixed with CT-B resulted in Peyer's patch and lamina propria T-lymphocyte populations which produced low IL-2 and IFN-γ but high levels of IL-4 and IL-5 (228).

However, other studies have shown that oral co-administration of a soluble or particulate antigen and CT primed animals for systemic Th1-type immune responses (delayed-type hypersensitivity) (229) and induced functional CTLs in the spleen (230). Again, the results from these studies suggest that oral immunization with heterologous antigen and CT as an adjuvant can differentially affect mucosal and systemic immune responses, inducing Th2-type immune responses in mucosal tissues and Th2 as well as Th1-type responses in the systemic compartment. Thus, not only the nature of the adjuvant, but also that of the antigen used, and the mucosal route of delivery can all influence whether Th1- and/or Th2-type responses develop.

CT and LT cause severe diarrhea in humans and account for the clinical manifestations of cholera and enterotoxigenic *E. coli* enteritis, and thus neither is suitable for use as an enteric adjuvant in humans. Early studies attempted but failed to dissociate diarrhoeagenicity from the adjuvanticity of these two molecules. For example, a non-toxic mutant, formed by making a single amino acid substitution in the ADP ribosyltransferase active center also lacked adjuvanticity when administered orally (231). However, mutants of LT formed by substituting a single amino acid either in or outside the ADP-ribosyltransferase cleft, retained adjuvanticity despite apparent lack of toxicity when administered by the intranasal route (108, 232–234). Moreover, two mutants of CT, which harbor single amino acid substitutions in the ADP-ribosyltranferase active center and completely lack ADP-ribosyltransferase activity and diarrheagenicity, remained effective adjuvants and were comparable to native CT when given parenterally or nasally (235, 236). We have ourselves found that recombinant CT-B and LT-B are also moderately adjuvant when co-administered with other protein antigens by the nasal route but not by the oral route (C. Czerkinsky, J. Holmgren, unpublished observations). Since enterotoxicity is the main obstacle to the use of native CT or LT as oral adjuvants, it will be interesting to determine whether enzymatically inactive CT and LT mutants have retained adjuvant activity when given orally.

Mucosal tolerance and anti-inflammatory vaccines

One of the primary goals in developing effective therapies against diseases caused by unwanted or tissue damaging inflammatory immune responses, is to specifically suppress or decrease to an acceptable level the intensity of untoward immune reactions without affecting the remainder of the immune system. Induction of tolerance in mature pathogenic T cells represents an ideal form of specific immunotherapy in the treatment of chronic inflammatory autoimmune disorders and allergies.

Three main modes of peripheral tolerance induction by antigens have been considered: parenteral administration of antigens, parenteral administration of antigen analogs which act as T-cell receptor antagonists, and mucosal administration of antigens or so-called "oral tolerance". Mucosal administration of antigen is in fact a long-recognized method of inducing peripheral tolerance. The phenomenon, often referred to as "oral tolerance" (because it was initially documented by the effect of oral administration of antigen), is characterized by the fact that animals fed or having inhaled an antigen become refractory or have diminished capability to develop an immune response when re-exposed to the same antigen introduced by the systemic route, e.g. by injection. This effect is especially pronounced for Th1 cell-mediated immune responses and is regarded as an important natural physiological mechanism whereby we avoid developing delayed-type hypersensitivity reactions to dietary and airborne antigens, and products from commensal microorganisms. Mucosally induced immunological tolerance can affect all types of adaptive immune responses, depending on the animal species, the age, the form and dose of antigen, and the route of mucosal administration (enteric, buccal, nasal, rectal, genital).

Mechanisms of tolerance after mucosal delivery of antigens

Mucosal uptake of antigens may result in the development of immunity or tolerance, or even both, the decision being taken in the epithelium or underlying lymphoid tissue and being mainly determined by the nature and physico-chemical form of the antigen.

Depending upon the dose of antigen administered, deletion or anergy of antigen-specific T cells and/or expansion of cells producing immunosuppressive cytokines (IL-4, IL-10 and TGF-β) (reviewed in (73)), may result in decreased T-cell responsiveness. It is interesting to note that the latter scenario involves cytokines that are also known to promote IgA isotype switching and IgA production, and is thus compatible with the observation that secretory IgA antibody responses and systemic

T-cell tolerance may develop concomitantly (237). Because tolerance can be transferred by both serum and cells from tolerized animals, it is possible that humoral antibodies (IgA?), circulating undegraded antigens or tolerogenic fragments and cytokines may act synergistically to confer T-cell unresponsiveness. Without excluding the above possibilities, another mechanism that may be considered could involve antigen-driven attraction of inflammatory T cells from the periphery into the mucosal microenvironment where they could be rendered anergic, functionally skewed, deleted or even ignored. This form of antigen-driven "anatomic deviation" would imply that inflammatory T cells activated in peripheral tissues exhibit a promiscuous migratory behaviour allowing their entry not only in systemic organs but also in mucosal tissues at the site of antigen uptake. The latter scenario has not yet been addressed experimentally but is compatible with studies showing that arthritogenic and diabetogenic T cells do express the cell surface mucosal integrin $\alpha4\beta7$ whose ligand, the mucosal addressin cellular adhesion molecule (MadCAM-1), is also expressed on inflamed pancreas and synovial tissues. In this respect, it is also interesting to note that oral tolerization with a prototype soluble protein antigen has recently been shown to differentially affect the production by peripheral T cells of the β (C-C) chemokines MIP-1α and MCP-1 (238), which are potent chemoattractants for T cells.

Irrespective of effector mechanism(s) involved, a major question that arises is where and how tolerance is induced, be it suppression, anergy, deletion, ignorance and/or anatomical deviation. To date, very little is known regarding the mechanisms governing induction of mucosal tolerance, and especially the intracellular pathways of entry of tolerogens, the nature of APC elements involved, their tissue localization, and the characteristics of signals transduced from such cells to responding T cells.

At variance with systemically administered antigens, antigens handled in mucosal tissues have already been subject to a variety of innate factors such as, e.g., proteases, acids, salts, *mucins*, that have altered their form prior to uptake. As a result of this "extratissular conditioning" different epitopes may be exposed and their uptake and/or processing may involve many different cell types.

The observation that mucosally induced systemic tolerance depends on an intact epithelial barrier (239, 240) suggests a central role for the epithelium. M cells have been shown to uptake a variety of particulate antigens such as viruses and bacteria, and to allow direct entry of invasive microorganisms in mucosal inductive sites. Such a pathway is thought to result in the induction of secretory IgA immune responses. Although the ability of M cells to serve as APCs appears to be poorly supported, these cells could still theoretically be involved in an aborative form of antigen presentation leading to tolerance induction. The role of absorptive epithelial cells, such as intestinal enterocytes, in tolerance induction has been underscored by several studies (reviewed in (241, 242)). Epithelial enterocytes express co-stimulatory molecules such as non-classical MHC class I (CD1d) molecules involved in antigen presentation to subpopulations of T cells and abnormal forms or levels of MHC class II molecules, leading to selective triggering of suppressive $CD8^+$ T cells and/or abortive presentation to $CD4^+$ T cells. In addition, epithelial enterocytes have also been shown to produce cytokines, such as IL-10 and TGF-β, which are particularly efficient at suppressing the inductive phase of $CD4^+$ T-cell-mediated responses.

All known types of classical APCs, including DCs, macrophages and B cells, and even mast cells, have been shown to populate mucosal tissues but, because of their heterogeneity and of the difficulty in isolating pure subpopulations of APCs from mucosal tissues, their respective role in inducing tolerance has not yet been elucidated. Although activated B cells and tissue macrophages are powerful APCs for memory Th cells, evidence suggests that antigen presentation by resting B cells results in T-cell tolerance (243, 244). It should however be noted that B cells activated *in vitro* with bacterial lipopolysaccharide (LPS), a prominent component of the normal mucosal microflora, are capable of inducing tolerance when injected into naive hosts (244). Although functional DCs have been identified in mucosal tissues such as the Peyer's patches, the mesenteric lymph, the intestinal lamina propria and the airway mucosa (reviewed in (245)), their role in activating rather than suppressing naive T cells has received strongest support. Thus, DCs freshly isolated from the airway mucosa preferentially activate Th2 cells but can also mature into APCs capable of activating Th1 (246). Thus, airway DC cells could play a pivotal role in the cross-regulation of Th1- and Th2-driven responses. However, recent experiments with splenic dendritic cells have shown a differential capacity of these DC subpopulations to activate T cells, suggesting the existence of regulatory and immunostimulatory DC subsets, supporting the emergence of the concept of tolerogenic DCs (247). Further, treatment with Flt3 ligand which is known to expand DCs *in vivo*, has been reported to enhance oral tolerance (248). Interestingly, LPS, which is known to cause the rapid exit of DCs, has also been shown to enhance tolerance induction (249). This information places strong emphasis on the site of entry and on the intracellular pathway of processing of antigens administered to a mucous membrane in the induction of tolerance and/or immunity and calls for the need to develop vaccine formula-

tions with intrinsic immunomodulating and cellular targetting properties.

Mucosal immunotherapy: potential and limitations

Since mucosally induced immunological tolerance is exquisitely specific to the antigen initially ingested or inhaled, and thus does not influence the development of systemic immune responses against other antigens, its manipulation has become an increasingly attractive strategy for preventing and possibly treating illnesses associated or resulting from the development of adverse immunological reactions against self and non-self antigens. The approach has been considered for preventing or treating hypersensitivities to common allergens (250–252).

The phenomenon of mucosally induced systemic tolerance has likewise been utilized to suppress immune responses against self antigens. It has thus been possible to delay the onset and to decrease the intensity of experimental autoimmune diseases in a variety of animal systems by mucosal deposition of auto-antigens onto the intestinal (by feeding) or the respiratory mucosa (by aerosolization or intranasal instillation of antigens) (reviewed in (73)). For instance, oral administration of collagen type II has been shown to delay the onset of autoimmune arthritis. Similarly, it has been possible to suppress an experimental form of autoimmune uveoretinitis by oral administration of the retinal S-antigen.

Although the above examples indicate that mucosal administration of foreign as well as self antigens offers good promise for inducing specific immunologic tolerance, the applicability of this approach in human medicine remains limited by practical problems. Indeed, to be clinically broadly applicable, mucosally induced immunological tolerance must also be effective in patients in whom the disease process has already established itself and/or in whom potentially tissue-damaging immune cells already exist. This is especially important when considering strategies of tolerance induction in patients suffering from or prone to an autoimmune disease or an allergic condition. Current protocols of mucosally induced tolerance have had limited success in suppressing the expression of an already established state of systemic immunological sensitization (253, 254). This may partly explain the disappointing results of recent clinical trials of oral tolerance in patients with multiple sclerosis and rheumatoid arthritis.

In addition, and by analogy with mucosal anti-infectious vaccines, induction of mucosal tolerance requires administration of massive amounts of antigens or prolonged administration of relatively smaller amounts of antigens which are then only effective in rather narrow dose ranges. As for conventional vaccines, efforts have recently been devoted to the construction of novel or improved formulations to induce mucosal tolerance. In this respect, the use of mucosal lectins endowed strong immunomodulating properties, such as cholera and E. coli heat-labile enterotoxin B subunits, as carrier for mucosal delivery of tolerogenic peptides has received considerable attention during the past five years as described below.

Cholera toxin B subunit as mucosal carrier-immunomodulating system for antipathological vaccination

Recent studies have shown that physical coupling of an antigen to CT-B led to unexpected effects: when given by various mucosal routes, CT-B induced a strong mucosal IgA immune response to itself and in some cases also to the conjugated antigen, but instead of abrogating systemic tolerance enhanced it profoundly (229). Based on this finding and on the results of other experiments with a variety of antigens (255), there is good reason to believe that such a system may be advantageous for inducing peripheral tolerance. First, it minimizes by several hundred-fold the amount of antigen/tolerogen and drastically reduces the number of doses that would otherwise be required by reported protocols of orally-induced tolerization. Second, but most important, this strategy appears to be applicable for suppressing the expression of an already established state of systemic immune sensitization. Here, we shall summarize the results of studies using this approach as a means to prevent or treat pathological immune responses associated with experimental autoimmune diseases, type I allergies, and allograft rejection.

Treatment of organ-specific autoimmune diseases

Mucosal administration of relevant autoantigens linked to CT-B could inhibit the development of clinical disease in animal models of experimentally inducible autoimmune diseases, such as allergic encephalomyelitis (256) and collagen-induced arthritis (257). In the latter model, nasal administration of a collagen type II–CT-B conjugate could inhibit disease progression, even when treatment was initiated after onset of clinically overt disease. Furthermore, oral treatment of female NOD mice with a CT-B–insulin conjugate could suppress type I diabetes (258), a model of spontaneous autoimmune disease, even when given as late as 15 weeks post-birth (that is at a time when all mice have evidence of insulitis). Taken together, these observations indicate that CT-B-driven mucosal tolerance can affect not only the afferent but also the efferent phase of systemic T-cell-mediated inflammatory responses.

Depending on the nature of the conjugated antigen, the route of administration (oral, nasal) of the conjugate, and the animal species used, this type of treatment variably affected the capacity of lymph node T cells to produce Th1 or Th2 cytokines.

The most striking observation in all three models of autoimmune diseases tested was the finding that treatment with CT-B-antigen suppressed leukocyte infiltration into the target organ. This suggests that the mechanisms governing induction of tolerance by feeding or inhaling CT-B-linked antigens may involve modifications of the migratory behavior of inflammatory cells.

Prevention of graft rejection

By coupling thymocytes to cholera B subunit and feeding this conjugate to mice, it has been possible to significantly prolong the survival of transplanted hearts in allogeneic mouse recipients (unpublished results). Recently, feeding CT-B-derivatized donor keratinocytes has been shown to prevent corneal allograft rejection in mice (259).

Prevention of type I allergies

The possibility of preventing type I allergic reactions by mucosal administration of a prototype allergen linked to a mucosal vector with intrinsic immunomodulating properties has also recently been examined in a mouse model of ovalbumin (OVA)-induced allergic reactions. Thus, mice nasally administered with OVA conjugated to *E. coli* LT-B prior to allergic sensitization showed suppressed skin DTH responses to OVA and also suppressed serum IgE antibody responses to the inhaled allergen (260). Further, these mice showed markedly decreased anaphylactic responses to intravenously administered OVA. Taken together, the latter observations indicate that under certain conditions, mucosal administration of soluble protein together with an immunomodulating mucosal vector can suppress both systemic Th1- and Th2-driven responses. The fact that the same type of regimen is also known to favor S-IgA responses in mucosal tissues makes this concept even more attractive since IgA is known to be non-phlogistic and could theoretically outcompete IgE for binding to a given allergen. However, it should also be pointed out that suppression of Th2-driven responses such as IgE antibody responses appears considerably more difficult to achieve than corresponding Th1 responses (e.g. DTH) in an animal already systemically sensitized to the allergen; in the latter situation, mucosal treatment with CT-B-conjugated allergen required prolonged administration of the conjugate and was effective only with certain allergens (C. Rask, J. Holmgren, C. Czerkinsky, unpublished observations).

Mucosal vaccines for simultaneous induction of anti-infectious and antipathological immunity

Somewhat surprisingly, vaccinologists in general and mucosal immunologists in particular have usually believed that a reciprocal relationship exists between induction of immunity and tolerance. The observation that mucosal immunity, which is typified by secretory IgA antibodies, may develop concomitantly with systemic immunological tolerance has led to the belief that vaccines against mucosal pathogens should primarily stimulate immunity without inducing tolerance. However, from a theoretical standpoint, the possibility to manipulate the mucosal immune system towards both immunity and tolerance appears rather attractive when considering strategies aimed at protecting the host from colonization or invasion by mucosal pathogens but also to interfere with the development of potentially harmful systemic immunological reactions against the same pathogens or their products.

The notion that immunological tolerance may provide the host with a protective mechanism against an infectious disease has been elegantly illustrated by recent studies in transgenic mice. Whereas mice from a susceptible (BALB/c) background develop an early Th2-driven IL-4 response and ultimately succumb to their infection with *Leishmania major*, mice rendered tolerant by transgenic expression in the thymus of LACK, a protective surface antigen of *Leishmania*, fail to produce this early response and resolve their infection (261). Very recently, tolerization of post-thymic, mature parasite-specific T cells could also be accomplished in the periphery after nasal administration of as little as 10 μg of *L. major* LACK antigen conjugated to CT-B (262). Such treatment markedly delayed the onset of lesion development in infected mice and reduced parasite burden in the skin and draining lymph nodes of infected mice.

Similar findings have also been observed in mice that had already been infested with the parasitic trematode, *Schistosoma mansoni* (263) and treated with a CT-B–parasite conjugate vaccine. Thus, nasal treatment of mice with *S. mansoni* glutathione S-transferase (GST) conjugated to CT-B suppressed granuloma formation and decreased parasite burden and egg deposition in the liver of infested animals. Protection with this nasal CT-B–GST vaccine was associated with decreased hepatic production of IFN-γ, IL-5 and IL-3 but apparently intact IL-4 production. Most importantly, such treatment could significantly prolong the survival of animals, even when initiated as late as 6 weeks after initial infection, that is at a time when liver granulomatous reactions are most pronounced.

While this type of approach has only been attempted in two parasitic diseases, there are obvious microbial diseases which could theoretically benefit from the concomitant induction of S-IgA immune responses and downregulation of local T-cell-driven immunopathology. Examples of such diseases include gastroduodenal ulcers caused by *Helicobacter pylori*, genital ulcers caused by papilloma viruses, broncho-pneumonitis

Table 1. Mucosal vaccines

Anti-infectious vaccines	Anti-inflammatory vaccines
Enteric infections	Chronic inflammation caused by microorganisms
Genitourinary infections	Autoimmune diseases
Respiratory infections	Allergies
Ocular, buccal and ear infections	Allograft rejection

induced by parainfluenzae viruses and respiratory syncytial virus, or chronic pelvic inflammaory disease, trachoma and urethritis caused by *Chlamydia pneumoniae*.

Other mucosal delivery systems for induction of tolerance

Most of the work published so far on induction of peripheral tolerance after mucosal administration of antigens has involved the use of free antigens and more recently that of antigens co-administered with CT-B or analogs. However, the possibility of developing improved tolerogenic formulations based on expression of selected antigens and/or immunomodulating cytokines and chemokines in appropriate vectors, e.g. lactobacilli, edible plants (264), or corresponding genes in plasmid DNA (265), are now being actively addressed.

Mucosally induced tolerance has the virtue of being a powerful natural and specific protective mechanism against adverse immune reactions that may result from mucosal intake of immunogenic matters. This property may be utilized to treat disorders associated with untoward immune responses to self and non-self antigens, such as certain autoimmune diseases, allergic reactions, and graft rejection (*Table 1*). Hence, mucosal immunomodulation via appropriate delivery of tolerogenic compounds may be where the future of anti-inflammatory vaccines lies.

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EXHIBIT

B

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Mucosal immunity and vaccines

Jan Holmgren[1] & Cecil Czerkinsky[2]

There is currently great interest in developing mucosal vaccines against a variety of microbial pathogens. Mucosally induced tolerance also seems to be a promising form of immunomodulation for treating certain autoimmune diseases and allergies. Here we review the properties of the mucosal immune system and discuss advances in the development of mucosal vaccines for protection against infections and for treatment of various inflammatory disorders.

The mucous membranes covering the aerodigestive and the urogenital tracts as well as the eye conjunctiva, the inner ear and the ducts of all exocrine glands are endowed with powerful mechanical and chemical cleansing mechanisms that degrade and repel most foreign matter. In addition, a large and highly specialized innate and adaptive mucosal immune system protects these surfaces, and thereby also the body interior, against potential insults from the environment. In a healthy human adult, this local immune system contributes almost 80% of all immunocytes. These cells are accumulated in, or in transit between, various mucosa-associated lymphoid tissues (MALT), which together form the largest mammalian lymphoid organ system[1].

The mucosal immune system has three main functions: (i) to protect the mucous membranes against colonization and invasion by potentially dangerous microbes that may be encountered, (ii) to prevent uptake of undegraded antigens including foreign proteins derived from ingested food, airborne matter and commensal microorganisms, and (iii) to prevent the development of potentially harmful immune responses to these antigens if they do reach the body interior. At variance with the systemic immune apparatus, which functions in a normally sterile milieu and often responds vigorously to invaders, the MALT guards organs that are replete with foreign matter. It follows that upon encountering this plethora of antigenic stimuli, the MALT must economically select appropriate effector mechanisms and regulate their intensity to avoid bystander tissue damage and immunological exhaustion.

Mucosal immune responses

The MALT represents a highly compartmentalized immunological system and functions essentially independent from the systemic immune apparatus. It is comprised of anatomically defined lymphoid microcompartments such as the Peyer patches, the mesenteric lymph nodes, the appendix and solitary follicles in the intestine, and the tonsils and adenoids at the entrance of the aerodigestive tract, which serve as the principal mucosal inductive sites where immune responses are initiated[2,3]. Small but numerous clusters of immature lymphocytes and dendritic cells have also been described in the subepithelial compartment of the mouse intestine and may represent sites of extrathymic lymphopoiesis[4,5]; such cryptopatches have not been found in humans, however. The MALT also contains diffuse accumulations of large numbers of lymphoid cells in the parenchyma of mucosal organs and exocrine glands, which form the mucosal effector sites where immune responses are manifested. Consistent with a high degree of compartmentalization, the MALT is populated by phenotypically and functionally distinct B cell, T cell and accessory cell subpopulations as compared with systemic lymphoid tissues, and has also developed strong restrictions upon lymphoid cell recirculation between mucosal sites.

As more extensively reviewed elsewhere[6], antigens taken up by absorptive epithelial cells and specialized epithelial cells (membrane, or 'M,' cells) in mucosal inductive sites can be shuttled to, or directly captured by, 'professional' antigen-presenting cells (APCs; including dendritic cells (DCs), B lymphocytes and macrophages), and presented to conventional $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells, all located in the inductive sites. Certain antigens may also be processed and presented directly by epithelial cells to neighboring intraepithelial T cells, including T cells with limited repertoire diversity ($\gamma\delta$ T cells and NKT cells). Immune responses in mucosal tissues are governed by the nature of the antigen, the type of APCs involved, and the local microenvironment. With most types of nonpathogen antigens (*e.g.*, food proteins), the 'default' pathway for mucosal DCs and other APCs seems to be to generate T helper 2 (T_H2) and various regulatory T cell types of responses[7]; this usually also results in active suppression of systemic immunity—'oral tolerance.' Antigens and adjuvants, including most pathogens, harboring motifs sensed by mucosal APCs as 'danger signals' (*e.g.*, Toll-like receptor (TLR) ligands), on the other hand, and proinflammatory conditions in general, favor the development of stronger and broader immune responses engaging both the humoral-secretory and cellular immunity effector arms and also do not lead to oral tolerance[2,6,7]. It has been widely assumed that the recognition of pathogens by TLRs on mucosal APCs was distinct from the response to the commensal flora, but recently it was found that microbial commensals are also recognized by TLRs under normal conditions, and that this interaction seems crucial for maintaining epithelial homeostasis in the gut[8].

The sensitized mucosal immunocytes, both B and T cells, leave the site of initial encounter with antigen (*e.g.*, a Peyer patch), transit through the lymph, enter the circulation and then seed selected mucosal sites, mainly

[1]Department of Medical Microbiology & Immunology and Göteborg University Vaccine Research Institute (GUVAX), Göteborg University, SE-405 30 Göteborg, Sweden. [2]INSERM Unité 721, Faculté de Médicine–Pasteur, F-06107 Nice Cedex 02, France. Correspondence should be addressed to J.H. (e-mail: jan.holmgren@microbio.gu.se).

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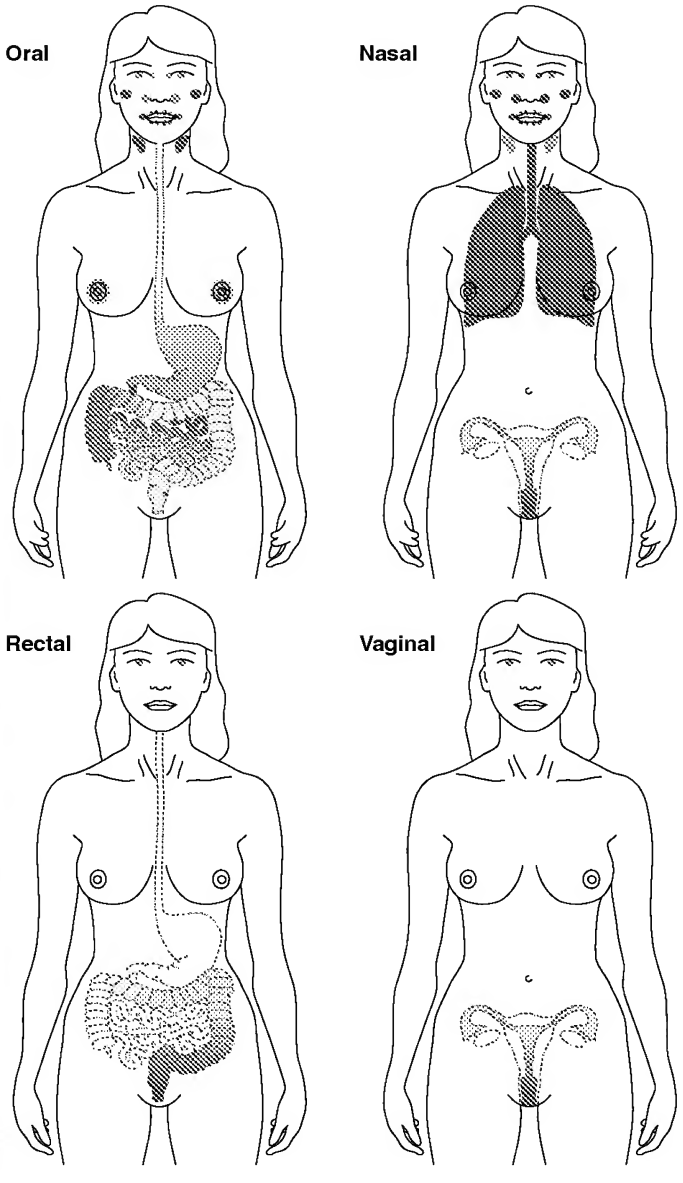

Figure 1 Expression of mucosal IgA immune responses after different routes of vaccination. The 'common mucosal immune system' is more restricted than previously thought. In humans, immunization studies with cholera toxin B subunit by different mucosal routes have clearly shown that the strongest response takes place at the directly vaccine-exposed mucosa and the second-best responses at adjacent mucosae or at specifically interconnected inductive-expression mucosal systems such as the gut-mammary gland link in lactating women. A notable exception is the fact that nasal mucosal immunization not only stimulates an immune response in the respiratory tract, but also can give rise to a strong genital-vaginal mucosal immune response. Shading indicates strength of response.

the mucosa of origin, where they differentiate into memory or effector cells. The anatomic affinity of such cells seems to be largely determined by site-specific integrins ('homing receptors') on their surface and complementary mucosal tissue–specific receptors ('addressins') on vascular endothelial cells[9]. In addition, chemokines produced in the local microenvironment promote chemotaxis toward mucosal tissues and regulate integrin expression on mucosal immunocytes, thereby controlling cell migration[10]. Of particular interest are recent studies indicating that mucosal DCs, in addition to presenting antigen to cognate T cells, can also influence their homing properties. Thus, mouse DCs isolated from mesenteric lymph nodes and Peyer patches, but not from spleen and peripheral lymph nodes, increase the expression of the mucosal homing receptor $\alpha4\beta7$ (refs. 11,12) and CCR9 (ref. 12), the receptor for the gut-associated chemokine TECK/CCL25 on memory T cells, and license effector/memory $CD8^+$ T cells to home preferentially to the intestinal epithelium. Notably, DC imprinting of gut homing specificity has recently been shown to involve retinoic acid, which is uniquely produced by intestinal DCs, but not by DCs from other lymphoid organs[13]. Taken together, these observations may explain the notion of a 'common mucosal immune system' whereby immunocytes activated at one site disseminate immunity to remote mucosal tissues rather than to systemic sites. At the same time, because chemokines, integrins and cytokines are differentially expressed among mucosal tissues, this fact may also partly explain why, within the mucosal immune system, there is a significant degree of compartmentalization linking specific mucosal inductive sites with particular effector sites (*e.g.*, the gut with the mammary glands and the nose with the respiratory and genital mucosae).

The compartmentalization within the mucosal immune system places constraints on the choice of vaccination route for inducing effective immune responses at the desired sites (**Fig. 1**). Thus, whereas oral immunization may induce substantial antibody responses in the small intestine (strongest in the proximal segment), ascending colon and mammary and salivary glands, it is relatively inefficient at evoking an IgA antibody response in the distal segments of the large intestines, tonsils or female genital tract mucosa[14–16]. Conversely, rectal immunization evokes strong local antibody responses in the rectum but little, if any, response in the small intestine and in the proximal colon[15–17]. Nasal or tonsilar immunization in humans results in antibody responses in the upper airway mucosa and regional secretions (saliva, nasal secretions) without evoking an immune response in the gut[18,19]; however, and of special interest for possible vaccination against HIV and other sexually transmitted infections, not only vaginal but also nasal immunization has been found to give rise to substantial IgA and IgG antibody responses in the human cervicovaginal mucosae[16,19,20]. Another notable finding, if it can be confirmed in humans, is that in mice, transcutaneous immunization may induce a mucosal immune response in the female genital tract[21]. It should also be borne in mind that the menstrual status of females may influence the intensity of immune responses in genital secretions[19,20].

Effector mechanisms

In addition to the barrier function, mechanical cleansing mechanisms and different chemical antimicrobial factors or defensins provided by the lining epithelium of different mucosal tissues, the mucosa contains a number of other cells of the innate immune system, including phagocytic neutrophils and macrophages, DCs, NK cells and mast cells. Through a variety of mechanisms these cells contribute significantly to host defense against pathogens[22] and for initiating adaptive mucosal immune responses.

The adaptive humoral immune defense at mucosal surfaces is to a large extent mediated by secretory IgA (SIgA) antibodies, the predominant immunoglobulin class in human external secretions. The resistance of SIgA to proteases makes these antibodies uniquely suited for functioning in mucosal secretions (**Box 1**). The induction of IgA against mucosal pathogens and soluble protein antigens is dependent on T helper cells[23,24], although IgA immunity to commensal flora may be thymus independent[25] and of low affinity[26]. In humans, transforming growth factor (TGF)-β and interleukin (IL)-10 in concert with IL-4 have been shown to promote B-cell switch to IgA and differentiation into IgA-producing cells[27,28]. In this regard, in addition to mucosal T cells,

which produce large amounts of IL-4, IL-10 and TGF-β, human mucoepithelial cells provide a major source of TGF-β and IL-10, suggesting that cooperation between neighboring lymphocytes and epithelial cells in the mucosal microenvironment is pivotal for programming preferential maturation of IgA-committed B cells.

Although SIgA is the predominant humoral defense mechanism at mucosal surfaces, locally produced IgM and IgG, and in the lower respiratory tract and in the genitourinary mucosa, serum-derived IgG can also contribute significantly to immune defense.

Mucosal cytotoxic T lymphocyte (CTL) responses have been described after oral, nasal, rectal or vaginal immunization[29,30], and recently also after transcutaneous immunization[31]. Mucosal CTLs have been shown to be crucial for the immune clearance of pathogens in several animal models of infection with enteric or respiratory viruses and intracellular parasites[32–34]. In most studies, wild-type or attenuated viruses and bacteria have been required to induce CTLs in mucosal tissues. There are, however, exceptions to this rule, inasmuch as use of certain adjuvants such as cholera toxin and related enterotoxins can promote mucosal CTL development when administered orally or nasally with soluble proteins and peptides[35,36]. Besides CTLs, interferon (IFN)-γ–producing $CD4^+$ T cells, induced either by the live pathogens or by mucosal immunization with inactivated vaccines in combination with cholera toxin or other mucosal adjuvants, have been found to be important for mucosal immune defense to both viral and bacterial infections; their protective mechanism(s), however, remain to be defined[37–39]. Thus, appropriate adjuvants or delivery systems, or both, may critically favor the induction of protective mucosal cellular responses, and this notion is of importance for developing mucosal vaccines against intracellular pathogens.

Regulatory mechanisms

The mucosal immune system has evolved a variety of mechanisms to achieve and maintain tolerance against self-antigens and against the plethora of environmental antigens present in the microflora, in food and among airborne matter. Studies in animal models have identified that mucosal tolerance can be achieved through different mechanisms, including activation-induced cell death, anergy and, most important, the induction of regulatory T cells[40]. Anergy of antigen-specific T cells has been reported after inhalation or ingestion of large quantities of soluble proteins[41], and deletion of specific T cells only after mucosal administration of massive, nonphysiological antigen doses[42]. Induction of regulatory cells after mucosal delivery of antigens has been reported in animal models for more than 25 years[43] and has received a major attention during the last few years given the potential of such regulatory cells as therapeutic agents in immune-mediated diseases.

In mice, four main types of regulatory T cells have been described: (i) antigen-induced $CD4^+$ T_H2-like cells that produce IL-4 and IL-10 and antagonize the activity of T_H1 effector cells[44]; (ii) $CD4^+CD45RB^{low}$ Tr1 cells that function through the production of IL-10 (ref. 45); (iii) $CD4^+$ or $CD8^+$ T cells producing TGF-β (T_H3 cells)[46]; and apparently most important, (iv) a population of naturally occurring $CD4^+CD25^+$ regulatory T cells (T_{reg} cells) that suppress proliferation through a cell contact–dependent mechanism[47,48]. Although anergic *in vitro*, the latter cells can be expanded in an antigen-specific manner *in vivo* after immunization[49,50]. Notably, these cells may also confer suppressor activity on other $CD4^+$ T cells by inducing the expression of the transcription factor Foxp3 and/or the major histocompatibility complex (MHC) class II–binding molecule LAG-3 in such cells ('infectious tolerance')[51,52]. Thereby, they may also provide a direct link between effector T-cell inhibition by T_{reg}, T_H3 and Tr1 cells. Thus, natural human $CD4^+CD25^+$ T_{reg} expressing the mucosal α4β7 integrin, when co-cultured with conventional $CD4^+$ T cells, induced Tr1-like IL-10–secreting T cells with strong suppressor activity on effector T cells, whereas another, α4β1-positive T_{reg} subset in similar cultures instead induced T_H3-like TGF-β–secreting suppressor T cells[51]. Recent evidence indicates that all of these different regulatory T cell types and mechanisms can be induced or expanded by mucosal administration of antigens leading to peripheral tolerance (oral tolerance; J.-B. Sun *et al.*, unpublished data).

Intraepithelial $CD8^+$ γδ T cells from the respiratory mucosa and from the small intestine have also been suggested to be involved in mucosal tolerance[53,54]. Despite their strategic location as the first immunocytes to encounter pathogens that have invaded an epithelial surface, the mechanisms of action of intraepithelial regulatory T cells are still largely unknown. The liver also seems to have a significant role in maintaining immunological silence to harmless antigenic material present in food. Regulatory T cells as well as NK and CD1-restricted NKT cells seem to contribute to the overall bias of hepatic immune responses toward tolerance and this bias may account for the survival of liver allografts and the persistence of certain liver pathogens such as hepatitis viruses[55,56]. Thus, protection of mucosal organs from autoagressive and allergic diseases seems to involve several layers of regulation.

Activation, expansion and survival of these various regulatory cells seem to be controlled by specialized types of APCs, especially tissue-specific DCs such as liver sinusoidal DCs, certain subpopulations of DCs in Peyer patches, mesenteric lymph nodes and small intestinal villi, or in the lungs[6,7,56,57]. Thus, mucosal DCs, depending on several major factors such as their tissue location, lineage to which they belong and the nature of maturation stimuli, can determine the character of the ensuing immune responses. Targeting regulatory or immunostimulatory mucosal DCs constitutes a major challenge to the development of adjuvants and vaccine formulations.

Mucosal vaccines against infections

The primary reason for using a mucosal route of vaccination is that most infections affect or start from a mucosal surface, and that in these infections, topical application of a vaccine is often required to induce a protective immune response. Examples include gastrointestinal infections caused by *Helicobacter pylori*, *Vibrio cholerae*, enterotoxigenic *Escherichia coli* (ETEC), *Shigella* spp., *Clostridium difficile*, rotaviruses and calici viruses; respiratory infections caused by *Mycoplasma pneumoniae*, influenza virus and respiratory syncytial virus; and sexually transmitted genital infections caused by HIV, *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and herpes simplex virus. These infections represent an enormous challenge for development of vaccines targeted to induce

BOX 1 SECRETORY IgA IN MUCOSAL DEFENSE

- Excessive production: >50 mg per kg body weight per 24 h
- Specific transport into mucosal secretions
- Resistant to host proteases
- Inhibits bacterial adhesion
- Inhibits macromolecule absorption (including uptake of or binding of allergens to mucosal target cells)
- Inhibits inflammatory effects of other immunoglobulins
- Neutralizes viruses (both extracellularly and within epithelial cells) and bacterial toxins
- Enhances nonspecific defense mechanisms (*e.g.*, lactoperoxidase and lactoferrin)
- Eliminates antigens in tissue through binding to IgA and subsequent polyimmunoglobulin receptor–mediated transport of immune complexes through epithelial cells

immunity that can either prevent the infectious agent from attaching and colonizing at the mucosal epithelium (noninvasive bacteria), or from penetrating and replicating in the mucosa (viruses and invasive bacteria), and/or that can block microbial toxins from binding to and affecting epithelial and other target cells.

A topical-mucosal route of vaccination seems to be crucial for protective efficacy against noninvasive infections at mucosal surfaces that are normally impermeable to serum antibody transudation, or passive passage across an epithelium. Gastrointestinal infections with *V. cholerae* or ETEC are examples of such infections, in which vaccine-induced protection is mediated mainly, if not exclusively, by locally produced SIgA antibodies and is associated with immunologic memory. In other instances, such as in *H. pylori* gastrointestinal infection and chlamydial or herpes simplex virus genital infections, the protective immunity is mediated mainly by mucosal $CD4^+$ T helper cells and possibly also $CD8^+$ CTL and NK cells[37–39]. When, on the other hand, the infection occurs at mucosal surfaces such as the respiratory and urogenital mucosae, which are more permeable than the intestine to transudation of serum antibodies, a parenteral route of vaccination may also be effective. The same may hold true for enteric infections in which the pathogen is first translocated across the epithelial barrier by intestinal membrane, or M, cells and then infects the basolateral side of the epithelium as is the case with *Shigella* spp., or causes disease only after multiplying and inducing inflammation in the submucosal lymphoid tissues (most salmonellae) and/or as for *Salmonella typhi* after further bacteremic spread in systemic tissues.

But despite the many attractive features of mucosal vaccination, it has often proven difficult in practice to stimulate strong SIgA immune responses and protection by mucosal administration of antigens. In fact, as yet only half a dozen of the vaccines that are currently approved for human use are administered mucosally (**Table 1**).

Table 1 Internationally licensed vaccines against mucosal infections

Infection and vaccine(s)	Route	Trade name (producer(s))
Polio		
Live attenuated vaccine (OPV)	Oral	Many
Cholera		
Cholera toxin B subunit + inactivated *V. cholerae* O1 whole cells	Oral	Dukoral (SBL Vaccin)
CVD 103.HgR live attenuated *V. cholerae* O1 strain	Oral	Orochol (Berna, SSVI)
Typhoid		
Vi polysaccharide	Deep subcutaneous or intramuscular	TyphimVi (Aventis)
Ty21a live attenuated vaccine	Oral	Vivotif (Berna, SSVI)
Rotavirus		
Live attenuated monovalent human rotavirus strain	Oral	RotaRix
Influenza		
Live attenuated cold-adapted influenza virus reassortant strains	Nasal	FluMist (MedImmune)

Oral polio vaccine. The oral polio vaccine (OPV) is the classical oral-mucosal vaccine. In addition to its enormous impact for reducing polio in the world, this vaccine has also served as a useful tool for elucidating fundamental aspects of mucosal immunity in humans[58]. Like the injectable inactivated polio vaccine (IPV), OPV produces antibodies in the blood that will protect against myelitis by preventing the spread of poliovirus to the nervous system. But, superior to IPV, OPV also produces a local SIgA immune response in the intestinal mucosa—the primary site for poliovirus entry and multiplication. This intestinal immune response can rapidly stop person-to-person transmission of wild poliovirus, making mass campaigns with OPV a powerful strategy for the global eradication of polio. At the same time, there is a low but real risk of reversion of OPV virus strains toward neurovirulence, which has led to the replacement of OPV by IPV in most industrialized countries. As the global eradication of polio is now approaching, concerns have been raised about the continued use of OPV in most developing countries after such eradication, and how to financially and logistically make it possible in these countries to use the safe but more expensive IPV instead.

Vaccines against enteric infections. Enteric infections resulting in diarrheal disease or typhoid fever remain a leading global health problem, causing an estimated 3 billion disease episodes and 3 million deaths annually in developing countries. Efficacious vaccines are now available against three of the main pathogens—*V. cholerae, S. typhi* and rotavirus—whereas vaccines are still lacking against the two other most important causes of disease, ETEC and *Shigella*.

Cholera vaccines. Almost half of all diarrheas are the result of enterotoxin-producing bacteria. Among these, *V. cholerae* causes the most severe disease and epidemic outbreaks. Previously used injectable cholera vaccines, which did not induce significant gut mucosal immune responses, afforded poor protection and have been abandoned. Recently, however, two improved oral cholera vaccines have become available. The most widely used of these vaccines (Dukoral), consisting of recombinantly produced cholera toxin B subunit (CTB) and inactivated *V. cholerae* O1 whole cells covering different serotypes and biotypes, has proven to be safe and stable and has in large efficacy (designed to measure the intrinsic effects of vaccination when given under ideal conditions) and effectiveness (designed to measure the vaccination effect under conditions likely to predict how well the vaccine will perform in public health practice) trials in Bangladesh, Peru and Mozambique conferred 85–90% short-term (first 6 months) and 60% long-term (first 3 years) protection against cholera[59–61] together with evidence of strong herd immunity[62]. Protection is mediated by local production of antitoxic and antibacterial SIgA antibodies in the gut. Through its CTB component, this vaccine also affords significant cross-protection against ETEC producing cholera toxin–like heat-labile toxin; ETEC is the most common cause of diarrheal disease both in children living in developing countries and among travelers to such countries. A simplified killed whole-cell cholera vaccine, without the CTB component, has also been produced, tested and licensed locally in Vietnam[63].

The second internationally licensed oral cholera vaccine is the live, attenuated vaccine CVD 103-HgR, containing a genetically manipulated classical *V. cholerae* O1 Inaba strain with a deletion in the gene encoding cholera toxin[59,64]. The vaccine has proven to be safe and has conferred 60–100% protection against experimental challenge with cholera organisms in North American volunteers. Based on this, CVD 103-HgR (Orochol) is licensed in several countries for use in travelers. But when the vaccine was tested in a large field trial in Indonesia, no significant protection was observed[65], and hence the usefulness of this vaccine in cholera-endemic areas remains to be established.

Typhoid fever vaccines. The old parenteral, killed whole-cell typhoid vaccine was effective, but it gave rise to severe local reactions and often fever. More recently, two safe and effective vaccines against typhoid fever have been licensed. The most widely used of these vaccines consists of purified capsular polysaccharide Vi antigen. This vaccine, which is given parenterally in a single dose, and is assumed to protect by way of serum antibodies, is well tolerated and, when tested in high-endemic countries, it gave 70% protection against typhoid fever during the first

12–18 months and 55% protection over a 3-year study period[66,67]. A locally produced Chinese Vi vaccine has afforded comparable (70%) protection[68].

The other licensed vaccine against typhoid fever is a live, attenuated oral vaccine, Ty21a, developed by chemical mutagenesis of a pathogenic *S. typhi* strain. Ty21a is well tolerated, and in schoolchildren in Chile three doses of the commercial enteric-coated capsule formulation were shown to provide 67% protective efficacy during the first 3 years of follow up[69]. The extent to which protection by this oral vaccine is mediated by local mucosal immunity in the gut or by systemic immunity is not known.

Rotavirus vaccines. Rotavirus together with ETEC is the most important cause of diarrheal mortality in infants and children below 2 years of age, causing 500,000–600,000 deaths annually, mostly in developing countries.

Most rotavirus strains belong to one of five antigenic groups. A quadrivalent vaccine based on a Rhesus monkey rotavirus strain equipped with human rotavirus genes was licensed for a short time (RotaShield), but was withdrawn after it was suggested that the vaccine caused intussusception (intestinal invagination)[70]. Recently, however, a new oral attenuated rotavirus vaccine (RotaRix) has been approved for human use in a few countries[71]. This single-strain vaccine is based on an attenuated human rotavirus strain and has shown good safety, and when tested in an efficacy trial in Brazil, Mexico and Venezuela, it conferred 61–92% protection against rotavirus hospitalizations. The vaccine is currently being further evaluated in Europe, Latin America, Africa and Asia. Another oral attenuated vaccine (RotaTeq) is also expected to be licensed soon in the United States and other countries[70]. This is a pentavalent reassortant bovine-human vaccine generated to contain human rotavirus genes for each of the main rotavirus serotypes. In Finnish infants, three doses of vaccine in different concentrations gave 59–77% protection against any rotavirus disease (T. Vesikari, H.F. Clark and P.A. Offit, personal communication).

Respiratory infections. Injectable vaccines against influenza and pneumococcal infections have been in use for a long time. Their main protective mechanism is the induction of serum antibodies, mainly IgG, which prevent systemic spread of the pathogen and which may also, through transudation, exert a local protective effect at the mucosal surfaces of the lower respiratory tract. Recently, however, a live influenza vaccine delivered by intranasal spray (FluMist), comprising cold-adapted, temperature-sensitive attenuated virus reassortant strains that are being adjusted to the antigenic needs for the actual influenza season, was licensed in the United States[72]. This vaccine induces an immune response that more closely resembles natural immunity than the response elicited by the injectable vaccine[73]. Both mucosal and systemic immunity contribute to resistance to influenza infection and disease: locally produced SIgA antibodies to virus surface hemagglutinin and neuraminidase are important for protection of the upper respiratory tract and corresponding serum IgG antibodies for protection of the lower respiratory tract and against viremia. Cell-mediated immunity, mainly against virus matrix and nucleoprotein antigens, does not protect against infection, but is important for clearance of virus and recovery from illness. The nasal vaccine induces significantly higher local IgA antibodies in nasal washings and local cell-mediated immunity but less high serum antibody titers than the injectable vaccine. Despite these differences in immune responses, the two types of vaccine have comparable protective efficacy (60–90%), and in elderly people, their combined use may increase the efficacy compared with the use of either vaccine alone.

Mucosal vaccines for immunotherapy

Immune responses are not fail-safe and may underserve or be excessive in protecting the host. As discussed above, several regulatory mechanisms to maintain control of mucosal immune reactivity are in place and are becoming increasingly appreciated as targets for manipulating immunopathologic responses. Mucosally induced immunological tolerance has become an attractive strategy for preventing and possibly treating illnesses resulting from the development of untoward immune reactions against allergens as well as self-antigens.

Vaccines against autoimmune diseases. Because induction of mucosal tolerance is antigen-specific but can be expressed in a nonspecific manner ('bystander suppression') through the production of suppressive cytokines by regulatory T cells in the inflamed microenvironment of the target organ, this approach has been utilized to suppress immune responses against self-antigens; however, it should be noted that bystander suppression remains to be documented in humans. It has been possible to prevent or to delay onset of experimental autoimmune diseases in various animal systems by feeding subjects selected autoantigens or peptide derivatives[40] (for example, in rodent models of autoimmune arthritis, type 1 diabetes, experimental autoimmune encephalitis, myasthenia gravis, autoimmune ear chondritis, autoimmune uveitis and autoimmune thyroiditis). In addition to the oral route, virtually all other routes of mucosal administration (nasal, buccal, rectal, genital) are also effective to induce tolerance, although to varying degrees. The dosage, the route and frequency of autoantigen administration have proven to be crucial. Thus, whereas low doses of nasally administered antigens favor expansion of regulatory T cells producing IL-10, low doses of orally administered antigen promote activation of $CD8^+$ and/or $CD4^+$ regulatory T cells producing TGF-β. Large doses of antigens seem to induce anergy of effector $CD4^+$ T cells, whereas massive doses can induce their apoptosis.

Although mucosal tolerance is usually effective in animal models for preventing inducible autoimmune diseases, its efficacy has been more variable and limited when utilized as an intervention strategy in animals in which the disease had already been induced or had spontaneously developed. This may explain, in part, the disappointing results of recent phase 3 clinical trials of oral tolerance in individuals with type 1 diabetes[74], multiple sclerosis[75] and rheumatoid arthritis[76], diseases in which there may be multiple target autoantigens that remain largely unknown.

A significant improvement has been achieved by coadministering immunomodulating agents to enhance the tolerogenic activity of autoantigens as well as allergens given orally or nasally. The most promising such agent is CTB, which when conjugated or coadministered with several autoantigens or allergens can markedly enhance tolerance induction in already sensitized animals and thereby effectively suppress progression of various autoimmune diseases[77–81]. Recently, a small phase 1-2 trial in individuals with Behcet disease, an autoimmune eye disease often associated with extraocular manifestations and abnormal T cell reactivity to a specific peptide within the human 60-kDa heat-shock protein, has shown the safety and clinical efficacy of treatment with an oral vaccine comprised of this specific peptide linked to CTB[82].

Antiallergy vaccines. Less well appreciated, this approach has given almost uniformly promising results in individuals with allergic disorders, to the extent that a World Health Organization expert group in a position paper recommended sublingual allergen-specific immunotherapy as the suitable treatment for allergic rhinitis in adults. The prevalence and severity of allergic diseases, in particular those affecting the respiratory tract, are increasing, and up to 20% of the population in developed countries are affected. Allergic rhinitis is one of the most common types of mucosal allergies, leading to asthma and early symptoms caused by a hypersensitivity response to airborne allergens such as pollens, dust mites, spores and animal dander. Type I allergy seems to result from

the selective activation of allergen-specific T_H2 $CD4^+$ T cells providing cognate help to IgE-committed B cells and resulting in hyperproduction of IgE. Specific inactivation of these allergen-specific T_H2 cells through clonal anergy, induction of T_H1-like cells, which are known to antagonize T_H2 cells (immune deviation), or induction of regulatory cells, are considered to be promising approaches for intervention in type I allergic diseases. Systemic allergen-specific immunotherapy by the injection of multiple (20 doses or more) small but increasing amounts of allergen can change a pre-existing allergic T_H2 immune response to a nonallergic T_H1 response. But this usually requires several years of treatment to be effective and the success rate is well below 50%. It is expensive and complicated, and also carries the risk of allergic and sometimes even life-threatening anaphylactic reactions.

The ability of secretory antibodies to interfere with the entry of allergens through the airway and the gut epithelium has been underestimated, despite the fact that SIgA is known to be noninflammatory and its daily output in external secretions exceeds that of IgG and by far that of IgE antibodies, which it could outcompete for binding to the target allergen. Furthermore, and at variance with systemic immunization, mucosal administration of antigens can induce SIgA antibody responses and, concomitantly, local and peripheral suppression of inflammatory responses. Because mucosal, especially oral or sublingual, vaccines are easier to deliver and safer than injectable vaccines, the concept of 'mucosal desensitization' has become increasingly attractive as an alternative to subcutaneous immunotherapy against type I allergies.

To date, more than 20 double-blind, placebo-controlled clinical trials of mucosal desensitization have been performed in individuals with allergic rhinitis and in individuals with bronchial asthma[83] (**Table 2**). Most studies have involved individuals with allergic rhinitis and have been based on oral and/or sublingual administration of allergen extracts from grass pollen, dust mites, cat, birch pollen, ragweed and *Parietaria*. Beneficial effects have been reported in the majority of these studies. In long-term studies of individuals with allergic asthma to house dust mites, oral-sublingual immunotherapy with allergen extract was efficient in reducing the frequency of asthmatic attacks and the use of antiasthmatic drugs[84]. Promising results have also been reported in individuals with atopic dermatitis[85].

Overall, the doses of allergen used in these trials and the frequency of allergen administrations have been rather high and, in the majority of trials, natural, and thus inherently heterogeneous, allergen extracts have been used. New techniques including allergen modification, allergen gene vaccination or peptide analogs in combination with selected adjuvants should further increase the safety and efficacy of mucosal immunotherapy in allergies and asthma[86].

Needs for improved mucosal vaccine formulations

The development of mucosal vaccines, whether for prevention of infectious diseases or for oral-tolerance immunotherapy, requires efficient antigen delivery and adjuvant systems. Ideally, such systems should (i) protect the vaccine from physical elimination and enzymatic digestion, (ii) target mucosal inductive sites including membrane, or M, cells, and (iii) at least for vaccines against infections, appropriately stimulate the innate immune system to generate effective adaptive immunity.

Mucosal delivery systems. A multitude of such vehicles have been developed, including various inert systems as well as live attenuated bacterial or viral vector systems[87–89].

Best known among the inert systems are various lipid-based structures with entrapped antigens, such as liposomes, immunostimulating complexes (ISCOMs) and so-called cochleates; different types of biodegradable particles based on starch or copolymers of lactic and glycolic acid; and different mucosa-binding proteins, including both classical plant lectins and bacterial proteins such as the binding subunit portions of cholera toxin or *E. coli* heat-labile enterotoxin, to which antigens have been linked either chemically or as gene fusion proteins.

Table 2 Successful sublingual allergy immunotherapy trials[a]

N trials	Allergen	*n* individuals	Adult or child	Duration
1	Ragweed	55	Adults	7.5 months
6	House dust mite	118	Adults and children	3–24 months
2	Olive pollen	43	Adults and children	2 months
5	*Parietaria*	79	Adults and children	6–24 months
5	Grass	154	Adults	3–18 months
1	Cat dander	20	Adults	3–5 months
1	Tree pollen	15	Adults	4–6 months

[a]As assessed by reduction in both clinical symptoms and use of antiallergic medication. Adapted from ref. 83. Duration indicates duration of treatment.

Among the many live bacterial vectors developed, two main categories can be distinguished: those based on attenuated pathogens such as *Salmonella typhi or S. paratyphi*, Bacille Calmette-Guérin or *Bordetella pertussis*; and those that use commensal bacteria, such as lactobacilli or certain streptococci and staphylococci. The initial use of vaccinia as the primary virus vector candidate has progressively been replaced by other poxviruses, such as canary poxvirus, and by adenoviruses. Several of the live vectors of both bacterial and viral origin have also been engineered to provide various cytokines to further stimulate or modulate the immune responses induced. But although many of these systems have shown promise in animal studies, there is still neither an inert nor a live vector approved for human use.

Promising results have recently been reported from the use of so-called pseudoviruses, or virus-like particles (VLPs). These are self-assembling, nonreplicating viral core structures, often from nonenveloped viruses, that are produced recombinantly *in vitro*. VLPs are cheap and easy to make, as well as highly immunogenic, and are therefore of commercial interest as viral vaccines in their own right. VLPs can, however, also be used as combined carriers and adjuvants both for foreign antigens expressed recombinantly on their surface, and for DNA vaccines carried within VLPs. VLPs are especially interesting from a mucosal vaccine point of view, as they offer the opportunity to use the natural route of transmission of the parent virus for vaccine delivery. Promising use of this principle, resulting in both SIgA and CTL mucosal immune responses and protection against mucosal pathogen challenge, has been reported from studies both in animals and in humans with VLPs from several mucosal viral pathogens including papillomavirus[90], calicivirus[91] and hepatitis E virus[92].

Mucosal adjuvants. When it comes to specific adjuvants, the best-studied and most potent mucosal adjuvants in experimental systems are cholera toxin and *E. coli* heat-labile enterotoxin[93,94], and much effort has been made recently to generate toxicologically acceptable derivatives of these toxins with retained adjuvant activity for human use (**Fig. 2**).

One such product is the completely nontoxic recombinantly produced CTB, which, depending upon the nature of the coadministered antigen, can be used to promote either mucosal immunity (mainly SIgA) to pathogens or peripheral anti-inflammatory tolerance to self-antigens or allergens[93]; the latter approach has recently also been tested clinically with promising results in individuals with Behcet disease[82].

Mutant heat-labile enterotoxin or cholera toxin proteins have also been made in which the toxic-active A (A1) subunit has been modified in various ways to remove the 'toxic' ADP-ribosylating activity, which leads to toxicity. In general, a loss of toxicity has been matched with a

corresponding loss of adjuvanticity, but a few proteins are available with significant adjuvanticity in the absence of detectable toxicity when given intranasally[95–97].

Yet another approach has been to prepare hybrid molecules in which the fully active cholera toxin A1 subunit has been linked to an engineered specific APC-binding protein derived from *Staphylococcus aureus* protein A (CTA1-DD)[98]. This specifically targets the molecule to B cells and has, in experimental systems, proven to be a very efficient and safe adjuvant for coadministered antigens when given intranasally. The incorporation of CTA1-DD and antigen into ISCOM particles may render the adjuvant effective for oral use[98].

Bacterial DNA or synthetic oligodeoxynucleotides containing unmethylated 'CpG motifs' (CpG ODN) represent another promising type of mucosal adjuvant. CpG ODN stimulate cells that express Toll-like receptor 9, thereby initiating an immunomodulating cascade. Although as yet mainly considered for systemic use, CpG ODN has been found after nasal, oral or vaginal administration to markedly enhance both innate and adaptive mucosal immunity in animal models[99,100], effects which were especially pronounced when CpG ODN was linked to the B subunit protein of cholera toxin[100].

Conclusions and perspectives

For many years, mucosal immunity and mucosal vaccines have attracted less than their due share of research and development, considering that most infections and environmental allergies have a mucosal portal of entry.

But in recent years, methodological advances allowing more intense study of mucosal immune responses have led to growing interest in both trying to better understand the specific features of mucosal as compared with systemic immunity, and to develop mucosal vaccines for preventing mucosal infections and for treating allergic or autoimmune diseases. Methods that facilitate the monitoring of mucosal immune responses in humans including infants and young children—the major target groups for vaccination against infectious diseases—have been developed, primarily for measuring secretory antibody responses. But practical assays for assessing mucosal T cell reactivity in clinical and in field settings are still scarce and methods for predicting efficacy of candidate mucosal immunotherapeutics in humans are lacking.

Mucosal immune responses in the humoral-secretory arm of the immune system develop earlier than systemic immune responsiveness, conferring a logistical advantage for mucosal vaccination in infants. On the other hand, it seems that mucosal tolerance develops much later, explaining, in part, the frequency (and often transient nature) of food allergies in young children. There is yet no precise knowledge regarding the ontogeny of the different mucosal regulatory cells for which selective targeting and activation by appropriate delivery systems and immunomodulating agents could be advantageous for preventing allergies and tissue-damaging inflammatory reactions.

Although effective oral-mucosal vaccines for human use are available, it is increasingly appreciated that the development of a broader range of mucosal vaccines, whether for prevention of infectious diseases or for immunotherapy of autoimmune, allergic or infectious inflammatory disorders, will require access to antigen delivery systems that can help present the relevant 'protective antigens' efficiently to the mucosal immune system as well as effective adjuvants to promote and direct the mucosal immune response toward the desired effect. Significant advances have recently been made in the development of improved mucosal vaccine delivery systems. Novel mucosal adjuvants with prospects for human use have also been designed.

Although these developments may promising useful mucosal vaccines, their usefulness in humans has yet to be established. It remains to be seen to what extent the safety and efficacy profiles established in animal models hold true in genetically diverse human subjects who also may differ significantly in their intestinal flora, nutritional status and previous immunological experience, all of which are factors that have been found to affect mucosal vaccine efficacy. Indeed, several mucosal vaccines, including oral live cholera vaccine and rotavirus vaccine candidates as well as OPV, have been found to work less well in developing country settings than in industrialized countries. The pandemic HIV infection problem presents additional challenges with regard to both vaccine safety and efficacy, especially for live attenuated vaccines. Although the main problems to date have dealt with lesser than expected efficacy of mucosal vaccines when tested in specific populations and settings, usually those prevailing in developing countries, it is also notable that two recently developed mucosal vaccines for human use—a live attenuated oral rotavirus vaccine and a nasal influenza subunit vaccine given together with (unmodified) *E. coli* heat-labile enterotoxin as adjuvant—were withdrawn after a short period because of adverse reactions, underlining the difficult and challenging task for all vaccines to combine vaccine and adjuvant efficacy with safety and public acceptability.

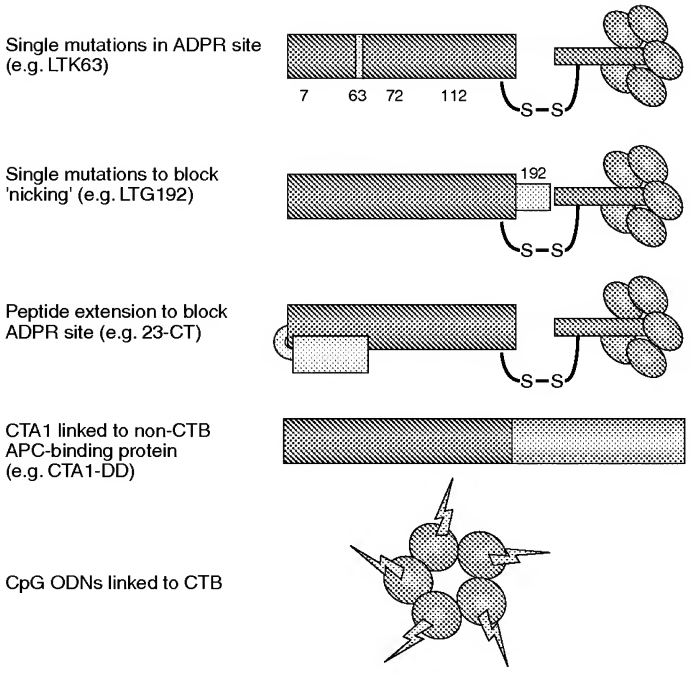

Figure 2 Different approaches toward development of mucosal adjuvants based on detoxified cholera toxin (CT) or *E. coli* heat-labile enterotoxin (LT). (ADPR, ADP-ribosylating; APC, antigen-presenting cell; CTB, cholera toxin subunit B.)

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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